ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 69, ART. 1 PAGES 1–254

BIOLOGICAL APPLICATIONS OF INFRARED SPECTROSCOPY

BY

ROBERT P. BAUMAN and CARL CLARK (*Conference Co-Chairmen*), E. AUGDAHL, A. A. BENEDICT, S. M. BIRNBAUM, E. R. BLOUT, J. CAROL, M. CHIANTA, R. CHILDS, L. DREISBACH, N. K. FREEMAN, M. M. GASTAMBIDE-ODIER, J. P. GREENSTEIN, R. G. GRENELL, R. S. HALFORD, R. N. JONES, R. J. KOEGEL, A. L. KOEVOET, U. LIDDEL, R. A. MCCALLUM, S. V. MASTRANGELO, L. MAY, A. NICKON, H. M. RANDALL, G. ROBERTS, M. ROGOFF, H. ROSENKRANTZ, H. P. SCHWARZ, D. W. SMITH, D. J. WHITTINGHAM, M. WINITZ, and D. L. WOOD.

NEW YORK
PUBLISHED BY THE ACADEMY
Sept. 7, 1957

THE NEW YORK ACADEMY OF SCIENCES

(Founded in 1817)

COUNCIL, 1957

President
ROSS F. NIGRELLI

President-Elect
BORIS PREGEL

Vice-Presidents

EDWARD J. KEMPF — HILARY KOPROWSKI

Recording Secretary
EMERSON DAY

Corresponding Secretary
FREDERICK C. NACHOD

Treasurer
ROBERT F. LIGHT

Elected Councilors

1955–1957

M. J. KOPAC
C. P. RHOADS

LLOYD C. MILLER
ELMER L. SEVERINGHAUS

1956–1958

DONALD B. KEYES
WARREN O. NELSON

CHARLES D. MARPLE
FREDERICK Y. WISELOGLE

1957–1959

GEORGE H. MANGUN
MINA REES

HAYDEN C. NICHOLSON
WILLIAM W. WALCOTT

Finance Committee

HARDEN F. TAYLOR, *Chairman*
GORDON Y. BILLARD — JOHN TEE-VAN

Executive Director
EUNICE THOMAS MINER

SECTION OF GEOLOGY AND MINERALOGY
M. HALL TAYLOR, *Chairman* — ANASTASIA VAN BURKALOW, *Secretary*

SECTION OF BIOLOGY
ALBERT S. GORDON, *Chairman* — LOUIS G. NICKELL, *Secretary*

DIVISION OF MYCOLOGY
M. L. LITTMAN, *Chairman* — KARL MARAMOROSCH, *Secretary*

SECTION OF PSYCHOLOGY
RALPH F. HEFFERLINE, *Chairman* — ELAINE GRIMM, *Secretary*

SECTION OF ANTHROPOLOGY
MARGARET MEAD, *Chairman* — DOROTHY CROSS JENSEN, *Secretary*

SECTION OF PHYSICS AND CHEMISTRY
ROBERT NEILSON BOYD, *Chairman* — JOSEPH GREENSPAN, *Secretary*

SECTION OF OCEANOGRAPHY AND METEOROLOGY
JEROME SPAR, *Chairman* — EDWIN L. FISHER, *Secretary*

SECTION OF MATHEMATICS AND ENGINEERING
PAUL HARTMAN, *Chairman* — NICHOLAS V. FEODOROFF, *Secretary*

Past Presidents

WILLIAM K. GREGORY — HORACE W. STUNKARD — HARDEN F. TAYLOR
VICTOR K. LAMER — M. L. CROSSLEY — M. L. TAINTER
WALTER S. ROOT

The Sections and the Division hold meetings regularly, one evening each month, during the academic year, October to May, inclusive. All meetings are held at the building of The New York Academy of Sciences, 2 East Sixty-third Street, New York 21, New York.
Conferences are also held at irregular intervals at times announced by special programs.

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 69, ART. 1 PAGES 1–254
Sept. 7, 1957

BIOLOGICAL APPLICATIONS OF INFRARED SPECTROSCOPY*

Conference Co-Chairmen: ROBERT P. BAUMAN and CARL CLARK

Consulting Editor: ROBERT P. BAUMAN

CONTENTS

*This series of papers is the result of a conference on *Biological Applications of Infrared Spectroscopy* held by the Section of Physics and Chemistry and the Section of Biology of The New York Academy of Sciences, December 7 and 8, 1956.

INTRODUCTORY REMARKS

By Robert P. Bauman
Polytechnic Institute of Brooklyn, Brooklyn, N. Y.

The infrared region has been known for about 150 years; spectroscopic identification of chemical substances has been practiced for about a century; and the application of the infrared region to problems of chemical analysis, as well as the recognition of "characteristic frequencies," is at least half a century old. Yet as late as 1909 F. Twyman of the Hilger Company, London, reported that he was strongly discouraged by workers in the field from venturing into the production of commercial infrared spectrometers. Indeed, even though commercial instruments were made available as early as 1913, very little tangible evidence of the growth of infrared spectroscopy appeared until the time of World War II. A comparison of the monograph by R. B. Barnes, R. C. Gore, U. Liddel, and V. Z. Williams, published in 1944, or even the 1949 publication of H. M. Randall, R. G. Fowler, N. Fuson, and J. R. Dangl, with the more recent publications, such as those of L. J. Bellamy or R. N. Jones and C. Sandorfy, provides a dramatic illustration of the growth of the practice of infrared spectroscopy over the last decade.

I should like to make only two general comments about this phenomenal growth and its significance. The first is well illustrated by many of the papers that are included in this monograph. In a field that is in practice so young, one should not feel that because a particular technique has not been successfully developed and reported in the literature it is an unpromising area of investigation. Only a very few years ago it was difficult to find any infrared spectroscopist who felt that it might be feasible to employ water as a solvent for infrared investigations. Within the last year, however, I have heard one talk entitled "The Advantages of Water as a Solvent for Infrared Spectroscopy," and I suspect that at least one of the papers in this publication could have used this as a subtitle. Only one extensive discussion of infrared spectra of solid inorganic compounds has appeared in the literature thus far. Several groups have already found the infrared region to be of great importance in investigating certain solids, such as proteins, the semiconductors, and glass and silica, but these investigations have thus far been concerned primarily with the gross features or with the regularities of these spectra. From my cloudy crystal ball I predict that within a year or two those who wish to study solid-state phenomena will discover that the vagaries of the infrared spectra of solids may be an invaluable probe into the mysteries of crystal lattices.

The second point is closely related to the first, for I think that a very large share of the credit for this growth rests with the unsung heroes of the infrared field: namely, the instrument manufacturers. In many fields of scientific instrumentation the development of a new instrument or method has prompted each of a dozen companies to assign a technician the task of designing an equivalent instrument in order to participate in the rewards without any real contribution to the work of development. Not only do the manufacturers of infrared

equipment lack this approach, but there seems to be an unusual degree of mutual understanding and respect between the spectroscopist and the manufacturer that forms the basis for a unanimity of purpose. We all know that the instrument companies are corporations established to show a profit at the end of each year. The method chosen for ensuring this, however, has been to make infrared spectroscopy such an indispensable part of modern scientific investigation that the market has grown beyond expectations. If one considers the variety of instruments and special attachments presently available, all produced by a very limited number of companies, I think it is clear that those who determine policy have truly taken an interest in our activities and have been most willing to cooperate in the projects that have been of interest to those of us in the laboratory. With such continued harmony it becomes difficult indeed to foresee what advances may come in another decade, although the contributors to this publication give an indication of some of the potentialities and the directions in which we may look.

UTILIZATION OF FRACTIONATION PROCEDURES WITH INFRARED ANALYSIS

By Harris Rosenkrantz
Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

The development of modern instrumentation has provided organic chemists and biochemists with several powerful weapons to aid in the isolation and identification of biologically important materials. The assurance that infrared spectroscopy would maintain its popularity stemmed not only from the continual refinement of instrumentation but also from the parallel improvement in fractionation techniques. These may be outlined as follows: chromatography, countercurrent distribution, preferential solvent extraction, sublimation, fractional crystallization, molecular distillation, dialysis, centrifugation, electrophoresis, diffusion, and freeze-drying. Specific enzymes have been used for resolving racemates or altering structure to modify physical properties. Finally, derivative formation—for example, the separation of ketonic materials by hydrazone formation or the use of acetylation to vary mobility rates on chromatograms—has been applied.

The utilization of these fractionation procedures has permitted the examination of the infrared absorption characteristics of almost every essential group of biological compounds. These include nucleic acids,[1-5] purines and pyrimidines,[6-10] proteins,[11-19] polypeptides,[20-22] amino acids,[23-29] lipides,[30-32] fatty acids,[33-35] carbohydrates,[36] porphyrins,[37] vitamins,[38-44] and the steroid hormones,[45-46] whose infrared characteristics have been extensively documented.

Of the various methods of purification listed, chromatography and countercurrent distribution have been the most widely applied to biochemical substances. It is the purpose of this presentation to discuss the methodology of these two fractionation procedures and to give illustrations of their application in the preparation of compounds for infrared spectroscopic analysis.

Description of Fractionation Procedures

A brief description of the historical evolution of the fractionation procedures to be discussed is in order. The history of chromatography can be divided into several branches, since variations of the system have given rise to adsorption column chromatography, partition column chromatography, paper chromatography (which may be adsorption or partition), and ion-exchange chromatography. Combination of the principles of paper chromatography and electrophoresis has provided the method of paper electrophoresis.

Column chromatography. In 1903 Tswett[47] reported that separation of plant pigments occurred when an extract was permitted to adsorb on a column of calcium carbonate. In the 1930's Diels and Rickert[48] utilized alumina as the adsorbent and, at the beginning of the 1940's, Gordon, Martin, and Synge[49] employed silica gel as the bulk material. Since then the following adsorbents have been used: magnesium carbonate,[50] magnesium silicate,[51] cellulose,[52-53] charcoal,[54] starch,[55] preparations made from earth,[56, 57] and rubber.[58] Solvents

vary from aqueous to the least polar organic solvents. Obviously mixtures of adsorbents or of miscible solvents can be used.

The separation of compounds achieved by adsorption chromatography is dependent upon the differences in the distributive behavior of each component between the solid phase and the flowing liquid. The stronger the affinity for the adsorbent and the poorer the solubility in the liquid phase, the longer the retention on the column. The application of alumina columns for the separation of steroids by Reichstein[59] and of starch columns for the resolution of amino acids by Moore and Stein[55] foreshadowed the potency of this tool in recent years.

In 1941 Martin and Synge[60] devised a theory for chromatography employing two liquid phases. This was the birth of partition chromatography and, incidentally, of countercurrent distribution. It was known that the partition coefficient of a compound in two immiscible liquids could be used as a physical constant of that compound. Since it was necessary to subject two structurally related substances to many partitions in individual separatory funnels for their resolution, an adsorbent column was used in order to create an infinite number of partition units: that is, a bulk material was saturated with one phase of the immiscible system, which was nonmobile, and the other phase was passed through the column. In theory, a distribution of each compound occurred at those particles of adsorbent containing the trapped nonmobile phase. No doubt some adsorption also occurred.

The bulk material used in partition column chromatography has included: silica gel, cellulose preparations, diatomaceous earth, paper impregnated with rubber latex, glass fibers, and vulcanized rubber powder. The two-phase liquid system may comprise only two solvents or mixtures of many solvents. Naturally, the type of compounds to be resolved determines the selection of adsorbent and solvents.

The sharpest separation of closely related structures is attained in column chromatography employing gradient elution.[61, 62] This technique was reported by Alm, Williams, and Tiselius[61] in 1952, and it consists of the elution of material by a continuously modified solvent. The development of the column is begun with a solvent in which the compounds are poorly soluble, and an external mixing arrangement slowly introduces increasing concentrations of a solvent in which the components are readily soluble. Mixtures of saccharides, amino acids, peptides, and steroids have been particularly amenable to this process.

Displacement occurs on adsorption columns; that is, a substance that has a greater affinity for the bulk material will displace a compound with a lesser affinity. In this manner the latter is pushed down the column and separated. Partridge[63] and others[64] have extensively investigated displacement development with synthetic ion-exchange resins. Adsorption by ion-exchange resins proceeds by the interchange of an ion from the solution with an ion of the same charge previously bound by the resin. Modern monofunctional resins contain one functional reactive group, which may be either negative or positive. These columns have yielded excellent results in the isolation of amino acids from urine or from protein hydrolyzates.

Paper chromatography. Both gradient-elution and ion-exchange resin

chromatography were developed after paper chromatography came into being. In 1944 Consden, Gordon, and Martin[65] realized the possibilities of separating substances on filter paper and detecting them by suitable color reactions. This approach also permitted the handling of micro amounts of material without difficulty. Since this discovery, both adsorption and partition procedures have been evolved. A simple example of the former is application of a urine extract to dry filter paper and elution with a polar organic solvent. It was found by Touchstone and Hsu[66] that the urinary pigments remain behind and that the formazan products of ketolic steroids can be extracted into methanol-ethyl acetate mixtures. The second technique, partition paper chromatography, involves impregnation of the filter paper with a nonmobile solvent and development with an immiscible solvent. Development may be halted after a suitable interval, and the resolved components may be detected directly on the paper by various methods. The success of partition paper chromatography in the separation and purification of very small quantities of naturally occurring products may be exemplified by the work of Consden, Gordon, and Martin on amino acids,[65] of Burton, Zaffaroni, and Keutmann[67] and Bush[68] on steroids, and of Calvin[69] and his colleagues in the elucidation of products in the photosynthetic cycle.

A paper chromatogram can be developed in one direction and then rotated 90° to be developed in a second direction; this is known as two-dimensional chromatography. The choice of solvent systems appears almost limitless.

Paper chromatography can be used as a preparative technique, as well as for analytical work. The compounds can be detected by suitable color reactions, by ultraviolet absorption, or by radioautography, and some success has been achieved by Kalkwarf and Frost[70] employing direct examination of paper strips exposed to infrared radiation. Instruments now available automatically feed paper strips through a densitometer, ultraviolet spectrophotometer, or Geiger counter, thereby recording the presence and quantity of substances on the chromatogram.

In order to inhibit the rate of mobility of nonpolar compounds, some investigators have impregnated the paper with alumina,[68] stearato chromic chloride,[71] vaseline,[72] or silicone,[73] thereby reversing the elution of the material; the polar compounds are eluted first instead of last.

A logical step was taken by Haugaard and Kroner[74] in 1948 when they applied a voltage across a paper chromatogram. The application of paper electrophoresis has been successful not only for the amphoteric amino acids, but also for steroids.[75]

Countercurrent distribution. At approximately the same time that Martin and Synge in Britain were elaborating the method of partition column chromatography and developing an extraction train for countercurrent liquid-liquid distribution, Craig and Post[76] in America were devising machines for carrying out many simultaneous partitions in units equivalent to cylindrical separatory funnels. Essentially, after a distribution was performed in the first tube containing the material, the apparatus could be revolved in such a manner that the lower phase of the initial tube (and successive tubes) could be exposed to a fresh upper phase and the upper phase simultaneously brought into contact

with a fresh lower phase. The process was continued until partition of material had taken place in all tubes.

The earliest apparatus permitted the transfer of about one gram of material over 24 tubes or plates. Since 1950, all-glass 200-plate instruments have been constructed. The liquid phases are transferred by decantation, and motor-gear systems have been designed to make the operation automatic.[77] Again, the general principle of fractionation by countercurrent distribution is based on the partition coefficient. One must select suitable immiscible solvent systems that give sufficient differences in the partition coefficients of the substances being concentrated or purified. The closer the partition coefficients, the greater is the number of transfers that must be effected.

Mathematical equations have been formulated for predicting the contours of a theoretical curve and for revealing in which tube the maximum concentration of material will occur.[78] The experimental curve's approximation to the shape of the theoretical Gauss curve is an indication of the extent of purification. The application of countercurrent distribution in the isolation and purification of antibiotics,[79] protein hormones,[80] steroids,[81] and vitamins[82] has firmly established this method as a sound fractionation procedure for biological compounds.

Utilization of Chromatography with Infrared Analysis

In addition to initiating purification, the techniques of chromatography afford some knowledge of chemical structure. This information can be deduced from mobility rates and from the reactions used for detecting the material. Such data may be very helpful in the interpretation of the infrared spectrum. For instance, a compound migrating more slowly usually contains more polar groups, such as oxygen or double bonds, as compared to a component moving more rapidly along the chromatogram. If detection is accomplished by a tetrazolium salt, a reducing group is indicated; ultraviolet absorption suggests conjugated systems; and so forth.

Adsorption column chromatography. Let us now illustrate the utilization of chromatography in connection with infrared analysis. After an incubation of Δ^4-pregnene-17α:21-diol-3:20-dione (Reichstein's Compound S) with rat liver homogenate, the steroid metabolites were extracted with acetone from the protein-precipitated solution.[83] Removal of the acetone was followed by partition of the fat-soluble material between 70 per cent methanol and ligroin. The ligroin eliminated a large quantity of contaminating lipides, including cholesterol. The steroids were recovered from the aqueous methanol by extraction into chloroform. The residue from the organic solvent was placed on a silica gel column, which then was developed with benzene, benzene-ethyl acetate, and ethyl acetate. Seventy-five 100 ml. eluates were collected and certain dried residues were subjected to infrared analysis. In some instances the material was crystalline. Other fractions required crystallization. Irrespective of form, the residue could be examined for infrared absorption characteristics.

In FIGURE 1 is shown a comparison of the infrared spectra (deposited films) of such a crystalline residue (a) with a carefully recrystallized sample (b). The material from the column was less pure, but the major component was

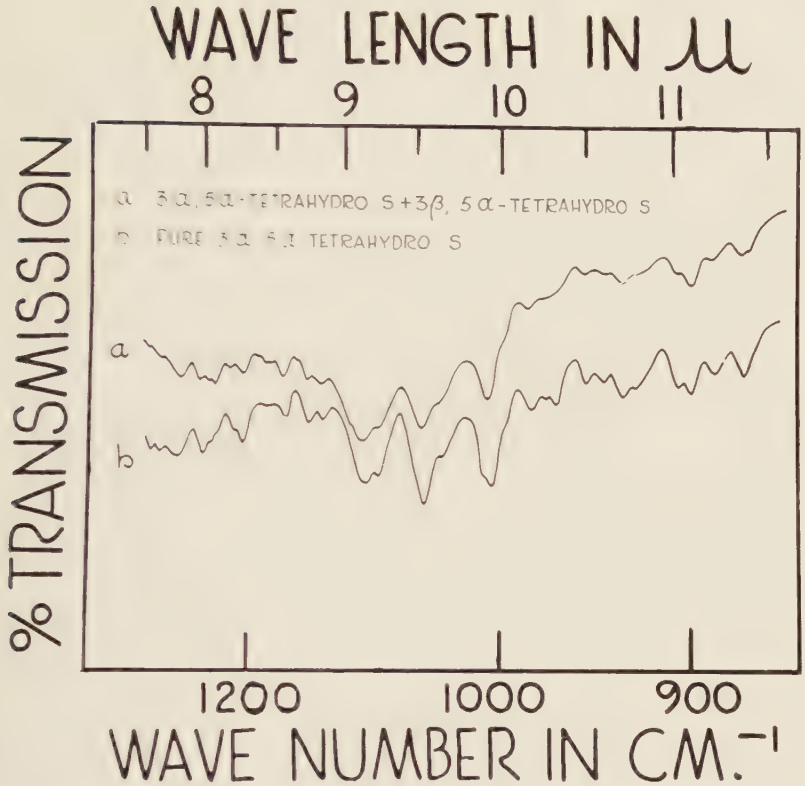


FIGURE 1. A portion of the infrared absorption spectra of (a) a mixture of 3α,17α,21-trihydroxypregnan-20-one and the 3β epimer and (b) pure 3α,17α,21 trihydroxypregnan-20-one.

identified as 3α,17α,21-trihydroxypregnan-20-one (3α,5α-tetrahydro S) by comparison with a purer sample of this compound. The presence of the contaminant was substantiated by identification of this component as 3β,17α,21-trihydroxypregnan-20-one (3β,5α-tetrahydro S) through formation of a digitonide. Although the adsorption column was apparently incapable of resolving these isomers, infrared examination prevented overlooking the presence of the 3β-hydroxy epimer.

In an investigation of steroid metabolites from the urine of normal and schizophrenic subjects, infrared analysis was used to compare corresponding fractions eluted from a silica gel column. The 3β-hydroxy steroids were of particular interest and were concentrated in the following way: acid hydrolysis, ether extraction, separation of the ketonic fraction by hydrazone formation, and removal of the 3β-ketosteroids by digitonin precipitation. Estimations of the quantity of material reactive in the Zimmermann test for 17-ketosteroids and the weight of the total residue in 24-hr. urines are given in TABLE 1. The last two columns, excluding those figures marked by a dagger, indicate that,

TABLE 1

ZIMMERMANN DETERMINATIONS AND DRY WEIGHTS OF 24-HOUR β-URINARY FRACTIONS FROM NORMALS

Zimmermann determinations (mg.)				Dry weight* (mg.)
Total neutral ketonic fraction	α fraction	β fraction	β fraction*	
11.41	12.94	—	0.71	4.1†
11.63	12.00	0.45	0.36	5.7†
9.11	8.26	0.31	0.23	7.2†
10.70	11.75	0.30	0.241	1.7
13.90	13.90	0.26	0.22	6.0†
6.45	6.24	0.195	0.15	1.6
8.50	7.75	0.21	0.17	3.1
10.50	10.10	0.196	0.165	1.5
14.20	15.05	—	0.45	6.4
20.65	19.70	1.725	1.38	4.9
7.46	6.30	0.275	0.22	4.6†
11.50	10.70	0.351	0.295	2.1
12.70	11.35	0.28	0.225	2.0
13.04	11.25	1.03	0.825	2.3
14.30	13.90	0.226	0.181	2.0
9.61	9.60	0.423	0.378	1.6
15.50	11.85	1.63	1.40	—
12.20	10.35	0.124	0.102	—
11.85	11.28	0.499	0.428	

* Material available after aliquot taken for Zimmermann estimation.
† α and β fractions separated by a method other than that of Butt *et al.*[92]

in urines from normal subjects, approximately 15 per cent of the residue may be steroidal in nature. The values marked by a dagger were obtained on residues derived from a less efficient digitonide separation. Approximately 10 per cent of the corresponding material exists in the urine from schizophrenics, as shown in TABLE 2. The β-ketonic fraction represented about 4 to 5 per cent of the total neutral ketonic steroids.

The residues containing the β-ketonic steroids were applied to a silica gel column, which was developed with benzene, benzene-ether mixtures, and methanol. Only three major fractions were obtained: a nonpolar eluate, a fraction eluted by 9:1 benzene-ether, and a methanol-soluble portion. Corresponding eluates from normal and schizophrenic urine did not yield to crystallization and were therefore submitted for infrared analysis. Despite the obvious presence of impurities in the spectrum of the 9:1 benzene-ether residue, dehydroepiandrosterone was identified. This metabolite is usually the major steroid in the β-fraction. The spectra of the other two eluates were very similar for the normal and schizophrenic fractions, although they were too distorted to allow the identification of particular compounds. This investigation was repeated, using paper chromatographic fractionation, and yielded the same results. FIGURE 2 shows the three fractions; zone II represents dehydroepiandrosterone. The system used could not separate dehydroepiandrosterone from epiandrosterone; here infrared analysis was helpful

TABLE 2
ZIMMERMANN DETERMINATIONS AND DRY WEIGHTS OF 24-HOUR β-URINARY FRACTIONS FROM SCHIZOPHRENICS

Zimmermann determinations (mg.)				Dry weight* (mg.)
Total neutral ketonic fraction	α fraction	β fraction	β fraction*	
10.30	5.96	0.106	0.095	2.6
8.56	8.34	0.103	0.087	1.0
6.29	6.45	0.087	0.070	1.2
7.50	7.43	0.138	0.11	1.9
8.06	8.45	0.143	0.115	1.7
6.69	6.75	0.119	0.095	1.9
7.72	7.40	0.27	0.23	1.5
7.91	7.65	0.23	0.185	1.2
7.98	8.40	0.152	0.134	1.1
11.90	—	—	0.36	—
11.10	12.00	0.875	0.70	2.1
10.50	8.77	1.26	1.005	—
9.45	6.75	1.23	0.99	—
12.70	10.95	0.204	0.175	—
25.65	25.50	2.16	1.85	—
6.42	6.15	0.146	0.12	—
4.35	4.40	0.119		—
6.86	6.52	0.158	0.136	—
9.44	8.70	0.441	0.362	

* Material available after aliquot taken for Zimmermann estimation.

in suggesting that if epiandrosterone was present in the urines, it occurred in much smaller quantities than dehydroepiandrosterone. None of the characteristic bands of the former could be recognized in the material from zone II. Although infrared analysis could not be more definitive in terms of structural identification, it showed that the β-ketonic fractions from normal and schizophrenic urines were qualitatively similar.

The next illustration involves the neutral ketonic steroids from the urine of an arthritic patient fed adrenosterone.[54] An initial fractionation was done on silica gel, and residues from pertinent eluates were subjected to paper chromatography. TABLE 3 outlines the solvents used on the adsorption column and the resolution of zones on the paper chromatograms. The enclosed zones yielded sufficient material for infrared identification and could be compared with material obtained directly from the column. Fractions 16 to 20, zone IV, contained $\Delta^{9(11)}$-androsten-3α-ol-17-one. Infrared analysis was particularly helpful in this case since, in the solvent system used, the saturated analogue, androsterone, has an identical mobility rate and the melting points are also very similar. It might be mentioned here that when an infrared spectrum reveals a cyclopentyl ketone in a residue that also gives the Zimmermann reaction, one has an excellent indication that one is dealing with a 17-ketosteroid.

Fractions 23 to 24, zone V, were shown to be composed of $\Delta^{9(11)}$-etiocholen-3α-ol-17-one by infrared analysis, and the curves were the same for the original oily material obtained from the column and the crystallized compound. Frac-

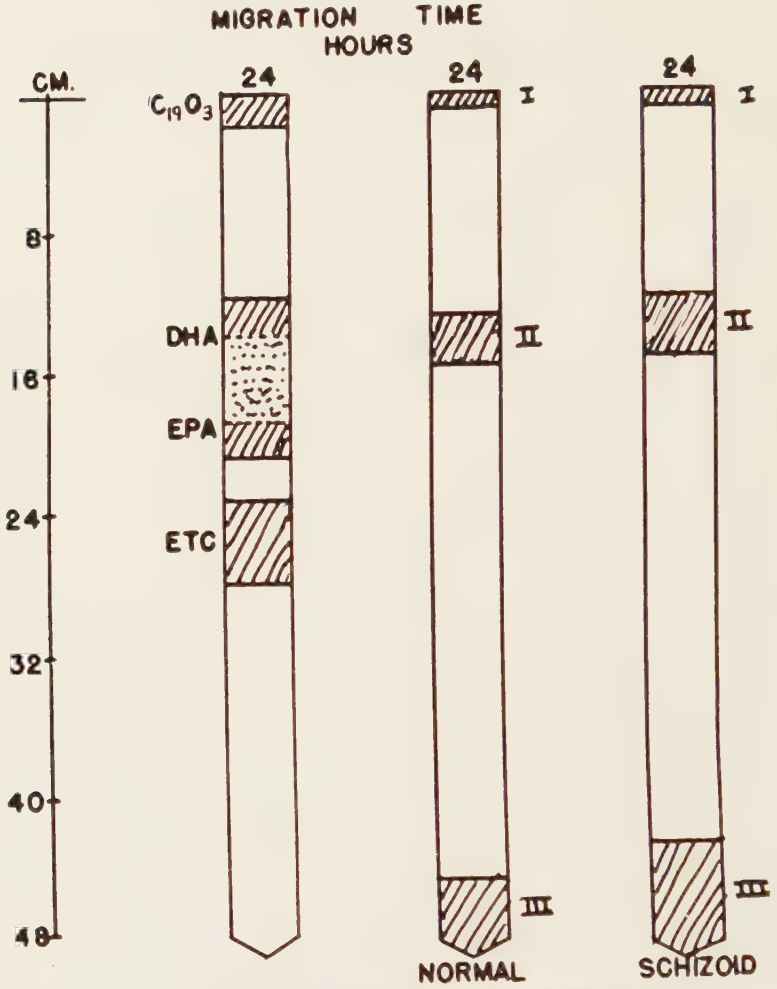


FIGURE 2. Paper chromatograms of urinary 3β-ketonic fractions partitioned in cyclohexane-propylene glycol. Reference compounds used were androstane-3α,11β-diol-17-one ($C_{19}O_3$), dehydroepiandrosterone (DHA), epiandrosterone (EPA), and etiocholan 3α ol 17-one (ETC).

tions 30 to 46 gave the following results: zone IX was 11β-hydroxyandrosterone, zone VIII was etiocholan-3α-ol-11,17-dione, zone VII was androstan-3α-ol-11,17-dione, and zone X was etiocholane-3α,11β-diol-17-one. The infrared identifications could usually be performed on noncrystalline material. A knowledge of the possible interpretations of the hydroxyl and carbonyl regions and of the 9 to 10 μ region, which reflects *cis trans* relationships of 3-hydroxysteroids, in addition to the information on mobility rates, permitted rapid differentiation and identification of these 11-oxygenated steroids.

Partition column chromatography. The previous illustration, plus considera-

TABLE 3

CHROMATOGRAPHY OF URINARY KETOSTEROID FRACTION

Fraction No.	Silica gel column: Solvent	Volume	Weight	Paper chromatograms of column fractions in ligroin propylene glycol: Steroid No.* I	II	III	IV	V	VIa	VIb	VIc	VII	VIII	IX	X	XI	Time
				R_T† 9.4–7.8	6.0–4.6	4.5–3.8	1.00	0.80–0.75	0.60	0.50–0.45	0.45‡	0.30	0.24	0.10	0.05	0.01	
		ml.	*mg.*														*hr.*
1–4	Bz§	300	83	++‖													3
5	"	50	40	+	+												3
6–7	"	100	37	+	++	++	+										3
8	"	50	14		+	++	+										3
9	"	50	6		+	++	+										3
10–15	Bz-EtAc 9:1	300	29			++	+										3
16	" 9:1	50	11			+	++	+									24
17	" 9:1	50	16				++	+	+	+							24
18	" 9:1	50	14				++			+							24
19–20	" 9:1	100	10				+	++	+	+							24
21–22	" 9:1	100	8				+	++		+							24
23–24	" 4:1	100	10					++		+		+					24
26–29	" 2:1	130	19					+		+	+	+					24
30	" 2:1	30	23									+	++	++	+		24
31	" 2:1	30	30									+	++	++	+		24
32	" 2:1	30	29									+	++	++	+		70
33–35	" 2:1	150	35									+	+	++	++		70
36–40	" 2:1	250	43									+	+	+	++	+	70
41–46	" 1:4	300	35									+	+	+	++	+	70

* Areas of steroids giving positive violet coloration with the Zimmermann reagent. Reprinted by permission from *The Journal of Biological Chemistry*.[84]

† R_T = relative mobility in cm. per hour.

‡ This steroid gave a positive reaction with silver diamine reagent.

§ Benzene.

‖ The plus signs denote the presence and intensity of steroid zones. The enclosed zones provided material for isolation studies.

ble experience with adsorption columns, indicates clearly that complex mixtures of steroid metabolites are more favorably separated by adsorption fractionation when it is followed by paper chromatography. Partition column fractionation appears to yield good resolution of closely related chemical structures. Here maximum efficiency of the partition column is obtained after preliminary purification on a silica gel or alumina adsorption column. Let us examine some illustrations of the use of the partition method.

Progesterone was perfused through human placenta, and the steroid metabolites were extracted with isopropyl acetate.[55] Partition between neohexane and 70 per cent methanol removed many of the undesirable lipides. A preliminary fractionation of the methanol residue on silica gel yielded the progesterone fraction in a benzene-ethyl acetate cut. The residue was transferred to a Celite column impregnated with aqueous methanol as the nonmobile phase, and the column was developed with cyclohexane. The progesterone material was resolved into two fractions on the partition column; this could not be accomplished by an adsorption silica gel column. At this stage infrared analysis identified one fraction as progesterone and disclosed an unusual situation in the carbonyl region for the other fraction. Although other information favored an α,β-unsaturated ketone and an intact C_{20}-carbonyl group, the infrared spectrum contained only one band at a frequency between a nonconjugated and a conjugated ketone. This suggested a direct influence on the Δ^4-double bond by another grouping. Oxygenation is the most likely transformation of steroids and, since hydroxyl absorption was absent, a 6-keto analogue of progesterone appeared likely. This was proved to be the case by comparison with the infrared curve of an authentic reference compound. 3,6-Diketo-Δ^4-cholestenone was also available, and its infrared spectrum also showed merging of all ketone functions into one band. The superior resolving power of the partition column and the use of infrared analysis made rapid identification of 6-keto-progesterone possible.

Another example of partition column chromatography involved the use of Hyflo Super-Cel containing 25 per cent ethanol as the stationary phase. Extracts of urine from guinea pigs fed cortisol were fractionated.[56] The mobile phase was alternately toluene, benzene, benzene-methylene chloride mixtures, methylene chloride-butanol mixtures, methylene chloride-ethyl acetate mixtures, and ethyl acetate. The chromatographic pattern is shown in FIGURE 3. Infrared analysis immediately identified the starting material, cortisol, in fractions 23 to 29. The peak between eluates 44 to 48 resisted crystallization, and samples were rechromatographed on the partition column and on paper. Both fractionation procedures yielded two fractions that could be identified as the C_{20}-reduced isomers, 20α-hydroxy and 20β-hydroxy, of cortisol.

The peak material between eluates 61 to 66 was subjected to a second chromatography and yielded 6β-hydroxycortisol. The ethyl acetate soluble material was shown by infrared analysis to be urea. Here the vibrations involving the nitrogen and carbonyl groups permitted a good guess as to the identity of the compound.

Paper partition chromatography. Although partition column chromatography has been impressive in resolving very similar compounds, preparation

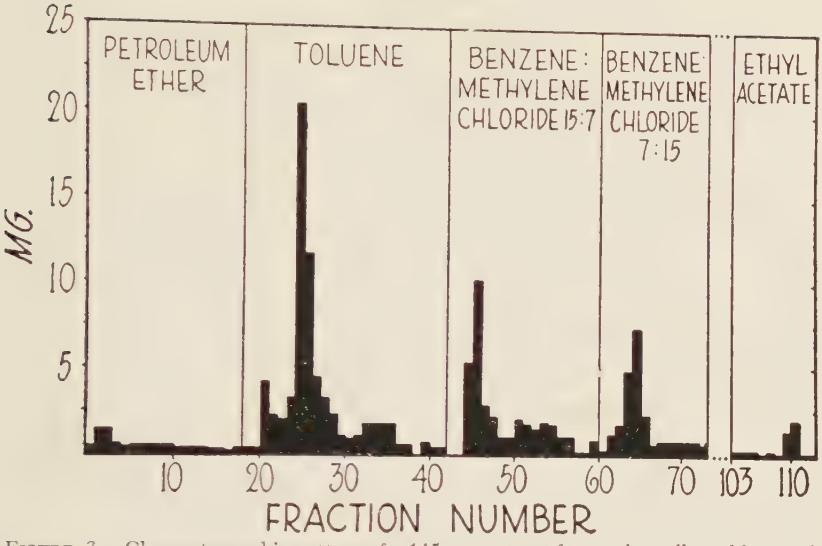


FIGURE 3. Chromatographic pattern of a 145 mg. extract from urine collected from guinea pigs fed 1 gm. of cortisol. Chromatography on Hyflo Super-Cel partition column with 25 per cent aqueous ethanol as the stationary phase. A total of 138 fractions of 40 ml. was collected by elution with various solvents as indicated. (Reproduced by permission from *The Journal of Biological Chemistry*.[86])

of the column has proven to be tedious and time-consuming. Paper chromatography not only dispenses with extra manipulations, but can be used analytically, or on a preparative scale. The following illustrations are representative of the utilization of this technique with infrared analysis.

Dehydroepiandrosterone was incubated with rabbit liver slices and the incubation medium worked up in a representative fashion for *in vitro* studies. Filter paper was impregnated with 50 per cent methanolic propylene glycol, and the developing solvent was toluene. In FIGURE 4 can be seen the localization of material by a reaction utilizing antimony trichloride in nitrobenzene.[88] This reagent has been particularly useful since it reacts very strongly with nonketonic steroids and gives different colors with different steroids. Only a section of the paper chromatogram is used for detecting the substances. Adjacent areas are cut out and eluted by methanol or methylene chloride and are individually rechromatographed. The overflow of the first chromatogram is captured in a receptacle and rechromatographed for a shorter interval of time. The two outside paper chromatograms show the distance of migration for dehydroepiandrosterone, Δ^5-androstene-3β,17β-diol, and Δ^5-androstene-3β,16α,17β-triol.

The preliminary chromatogram revealed three distinct (I, II, III) and two indefinite (IIa and IV) zones (FIGURE 4). Rechromatographing showed many components in zone I, two compounds in zone II, IIa, one metabolite in area III, and one in area IV, still contaminated with some III. The overflow contained two reactive materials, V and VI. Samples of corresponding unreacted areas were investigated in the infrared region initially without crystallization. Zone VI was dehydroepiandrosterone, III was Δ^5-androstene-3β,17β-

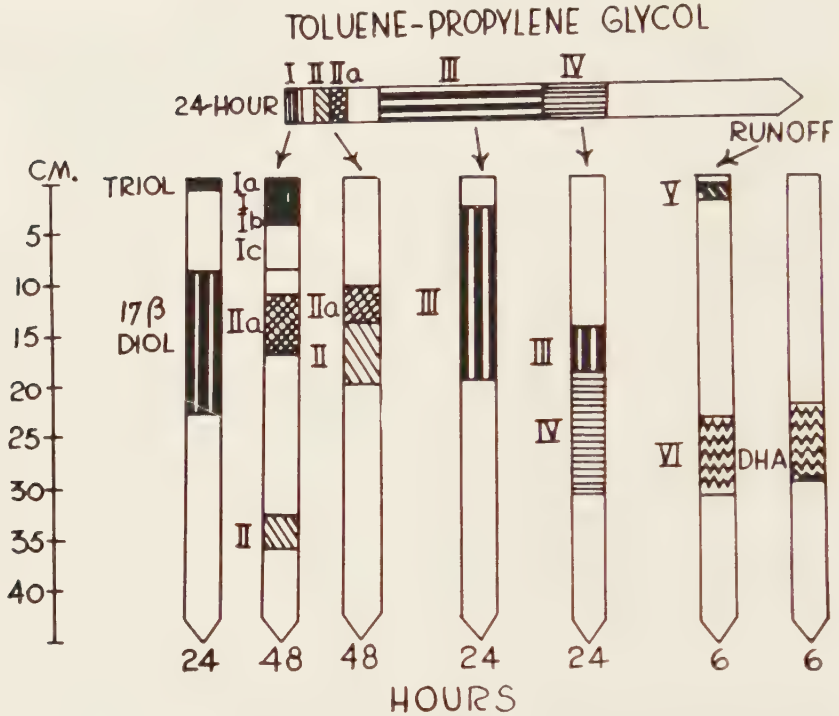


FIGURE 4. Paper chromatographic fractionation of a residue from a methylene dichloride extract of rabbit liver homogenate incubated with dehydroepiandrosterone. Reference compounds used were Δ^5-androstene-3β,16α,17β-triol (TRIOL), Δ^5-androstene-3β,17β-diol (17β DIOL), and dehydroepiandrosterone (DHA).

diol, and IV a mixture of the latter and its 17α-isomer. Examination of material from the area in IV furthest from III showed practically no contamination with the 17β-analogue. The other metabolites have not been identified, since very little material was available for further studies. The infrared spectra indicated that some of these components were ketonic and others nonketonic. The mobility rates suggested that hydroxylation to an O_3 or O_4 had occurred, accounting for zones I and II and related areas.

A second illustration concerns the identification of Krebs-cycle intermediates in rabbit skeletal muscle. Skeletal muscle was homogenized in ethanol, and aliquots of the supernatant were put on paper. An ascending chromatogram was developed in an ether-formic acid-water system.[89] In FIGURE 5 is depicted such a chromatogram and a chromatogram of a mixture of reference compounds. The unreacted area between citric and malic acids from the muscle extract was eluted in aqueous alcohol, dried, and examined in the infrared region. The spectrum indicated a mixture of citric and malic acids (FIGURE 6).

Utilization of Countercurrent Distribution with Infrared Analysis

It has become obvious that application of several chromatographic procedures affords a potent method for complete purification of substances. A com-

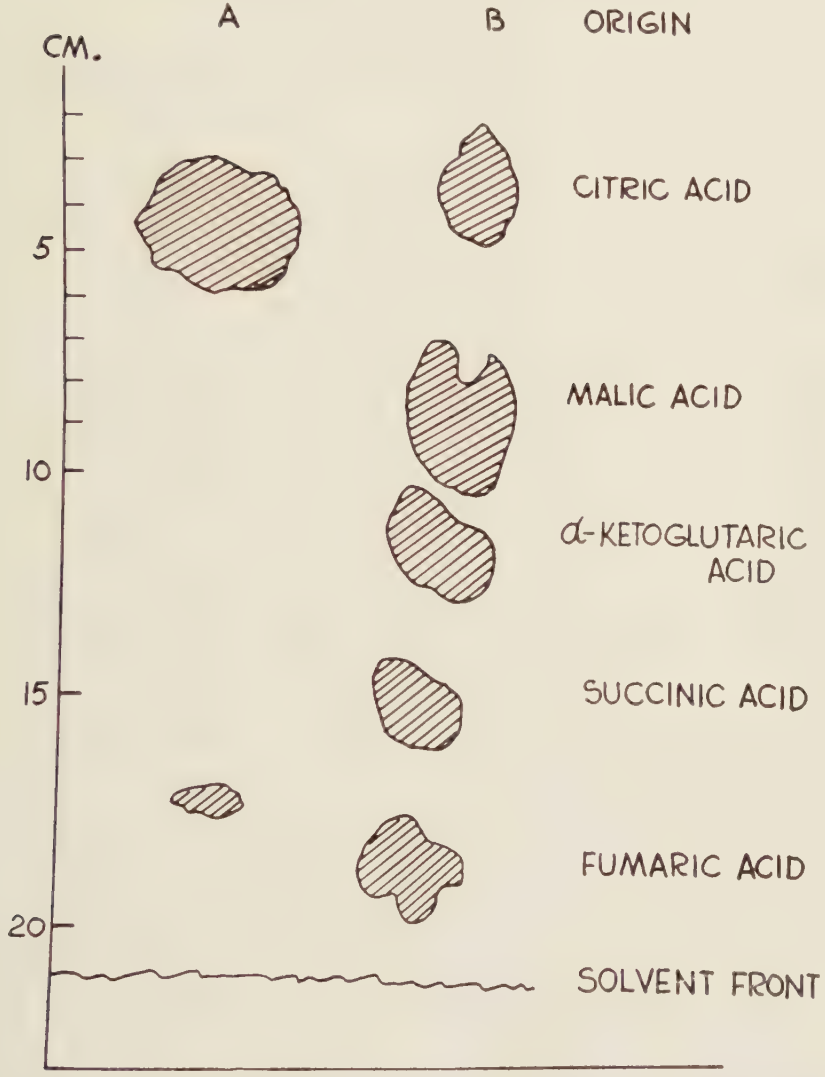


FIGURE 5. Ascending paper chromatography of Krebs-cycle intermediates in an ether-formic acid-water (13:3:1) system for 5 hr. (A) An aliquot of an aqueous ethanolic extract of rabbit skeletal muscle; (B) a mixture of standard organic acids.

bination of several fractionation techniques also enhances the efficiency of countercurrent distribution. Preliminary purification or repartition of particular concentrates yields excellent results with the Craig-Post type of apparatus. Infrared spectrometry may be applied after each step of the fractionation in order to evaluate the extent of separation achieved.

Antistiffness factor—stigmasterol. The next illustration is an outstanding example of the use of countercurrent partition in conjunction with infrared

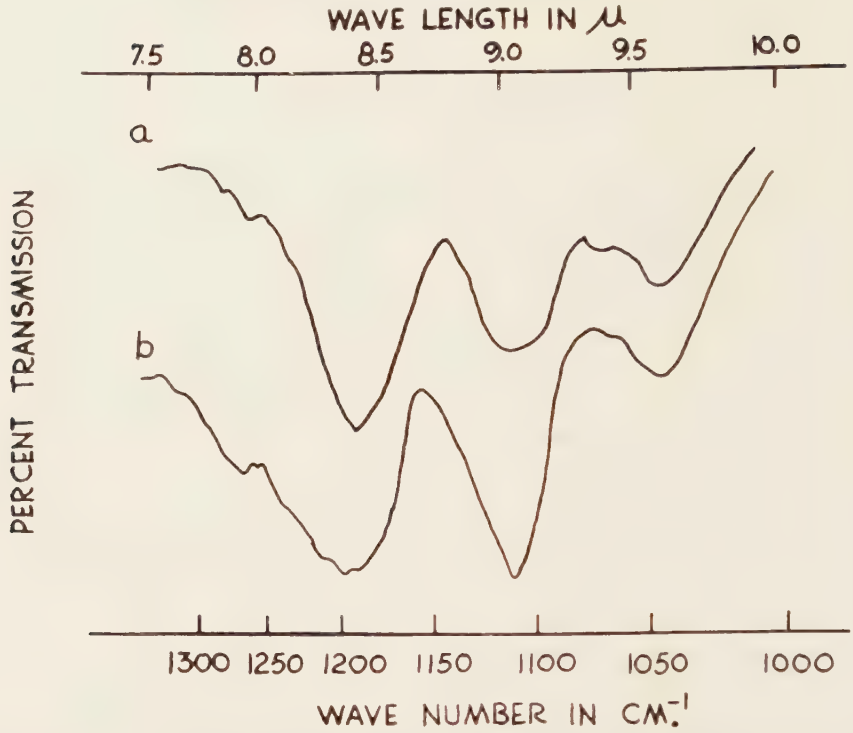


FIGURE 6. A comparison of infrared absorption spectra of (a) isolated organic acid mixture from skeletal muscle and (b) an authentic mixture of citric and malic acids.

analysis. The assignment was to identify a factor in a purified sterol concentrate from cane sugar that relieved joint stiffness in guinea pigs.[20] In preliminary work a positive Liebermann-Burchard test, indicating sterols, and a faintly positive Rosenheim test, suggesting the presence of some $\Delta^{5,7}$-diene steroid, were obtained. The latter was confirmed by characteristic ultraviolet absorption and a slightly positive diazo coupling test, which is a test for nonketonic steroids with a conjugated double-bond system. On the basis of the extinction coefficient of known $\Delta^{5,7}$-diene steroids, the concentration of the diene impurity was estimated to be 15 per cent.

The infrared spectrum of the purified concentrate contained a band near 3400 cm.$^{-1}$, which was assigned to a hydroxyl group, since naturally occurring steroids with NH groups were not known. Absorption near 1700 cm.$^{-1}$ indicated the presence of carbonyl and unsaturated groups. Two strong bands that appeared near 730 cm.$^{-1}$ were assigned to a nonsteroidal component on the basis of observations on a large number of steroid curves.

All this information indicated a mixture of at least three compounds: a sterol without a conjugated system, a diene sterol as a minor constituent, and a nonsteroid absorbing near 730 cm.$^{-1}$ Therefore, the concentrate (30 mg.) was

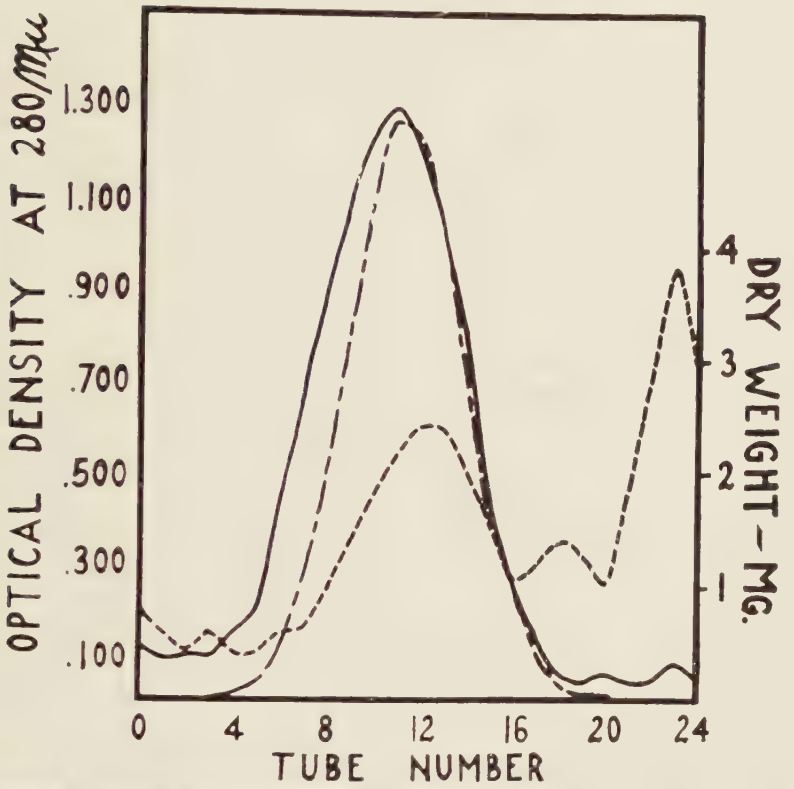


FIGURE 7. Countercurrent distribution in methanol-isooctane of a purified concentrate containing the antistiffness factor: absorption at 280 mμ (————); dry weight of distributed material (– – – –); theoretical curve based on a partition coefficient of 0.9 — — — — —[1].

subjected to a 24-plate distribution in isooctane-methanol. This partition is shown in FIGURE 7, in which tube number is plotted versus ultraviolet absorption and weight. Both analyses permitted the separation of the partitioned concentrate into fraction A, tubes 0 to 10; fraction B, tubes 11 to 19; and C, tubes 20 to 24. The concentration peak of the $\Delta^{5,7}$-diene material appeared in tube 11, and a theoretical curve indicated the presence of a mixture. The dry-weight determination demonstrated that 45 per cent of the weight was in B, 35 per cent in C, and 20 per cent in A.

The infrared spectra are shown in FIGURE 8. It can be seen that fraction A was free of C; the latter accounted for the absorption near 730 cm.$^{-1}$. Fraction A contained the hydroxyl group. Some carbonyl contaminant was evident near 1720 cm.$^{-1}$ (presumably C) while absorption near 1670 cm.$^{-1}$ favored an isolated ethylenic grouping. Fraction B was an unresolved mixture of A, C, and the diene. Sublimation of fraction B yielded additional A. During the

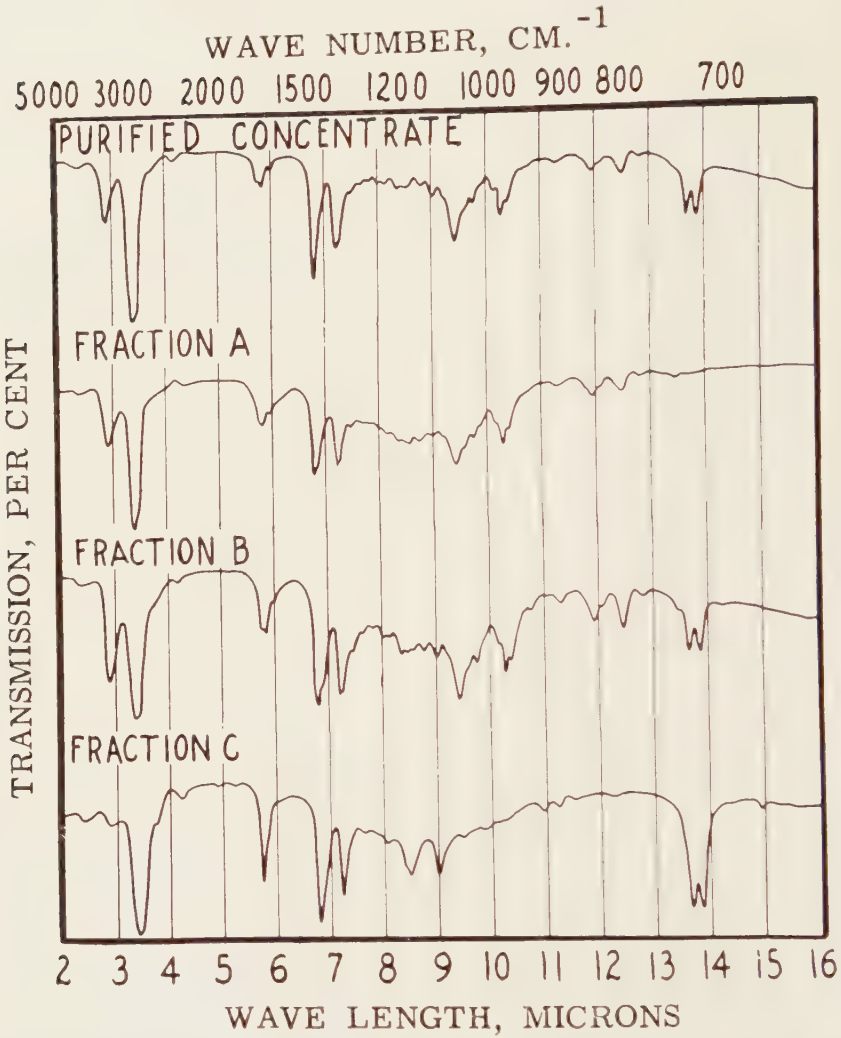


FIGURE 8. Infrared absorption spectra of a purified concentrate from cane sugar and of three fractions separated by means of a 24-plate countercurrent distribution. (Reproduced by permission from Interscience Publishers, Inc.[45])

application of the fractionation procedures, a catalogue of infrared spectra of pertinent steroids was being prepared and led to the identification of fraction A, the antistiffness factor, with stigmasterol. A similar conclusion was simultaneously reached by another group employing chemical methods of identification.

Identification of tocopherol compounds. The isooctane-methanol partition was also applied in an attempt to isolate and identify tocopherol compounds (vitamin E) in feces.[57] The partition coefficients of α-tocopherol and of its

oxidation product, α-tocopherylquinone, were quite similar, but characteristic ultraviolet absorption[41] could distinguish the quinone (268 mμ) from the phenol (294 mμ). The reduction product of the quinone, tocopherylhydroquinone, absorbed at a wave length (287 mμ) between the other two and had a significantly different partition coefficient. These three compounds could easily be differentiated on the basis of their infrared spectra, and it was only necessary to obtain suitably purified fractions for identification.[39, 40]

In one study, tocopherylquinone was fed to a subject, the feces dried on a steam bath, and an isooctane extract made in a Waring Blendor.[52] Three approaches to fractionation were made: (1) removal of many lipides by precipitation in ice-cold methanol; (2) a preliminary partition using 8 separatory funnels and selecting the center tubes; and (3) employment of a chemical procedure for reduction of the quinone to the hydroquinone and reoxidation back to the quinone, a procedure that purified tocopherylquinone. The pertinent fractions from each preliminary method were then distributed in a 24-plate Craig apparatus. Best results were obtained by procedure 3. The partition was determined by plotting the quinone absorption at 268 mμ against tube number (FIGURE 9). The absorption at 298 mμ was low. After conversion of the quinone to the phenol by reductive cyclization, the 268 mμ absorption

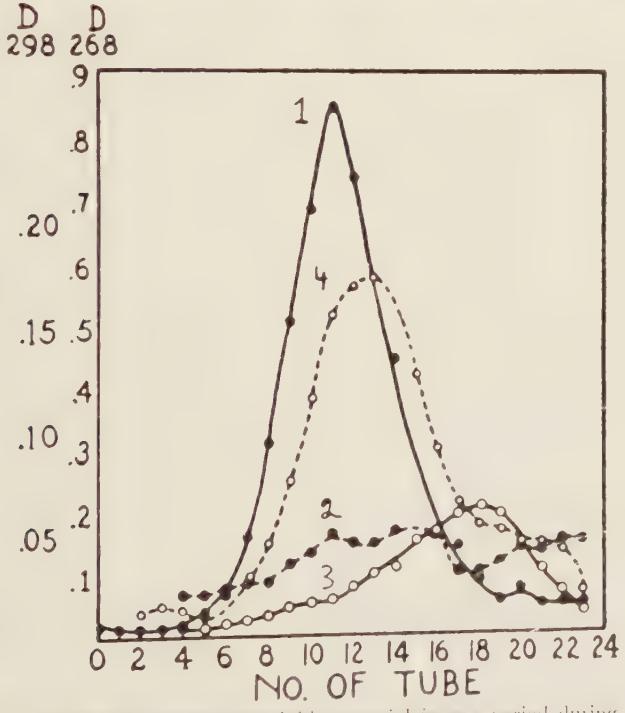


FIGURE 9. Distribution of the fat soluble material from a period during which tocopherylquinone was fed. Before cyclization, 268 mμ absorption (curve 1), 298 mμ absorption (curve 2); after cyclization, 268 mμ absorption (curve 3), 298 mμ absorption (curve 4). (Reproduced by permission from *The Journal of Biological Chemistry*.[52])

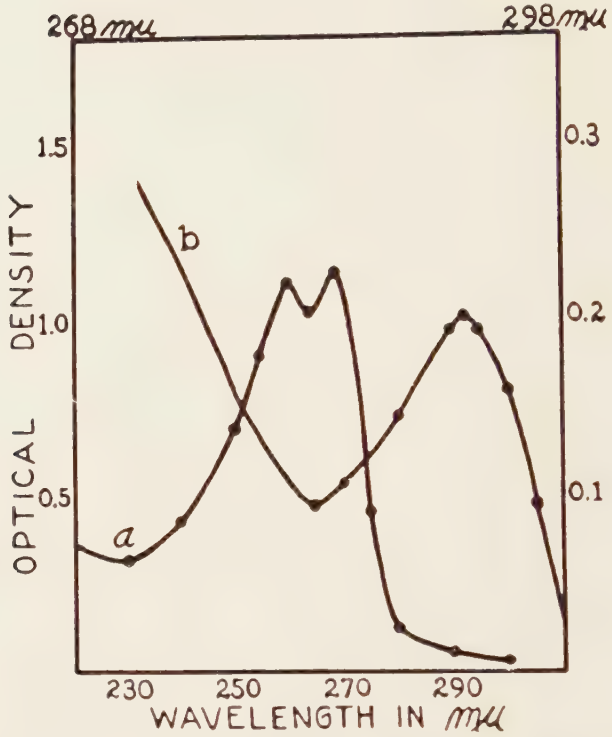


FIGURE 10. Ultraviolet absorption spectra of partitioned fecal matter from the tocopherylquinone period. (a) The material from tube 11 (see FIGURE 9) before cyclization and (b) after cyclization to tocopherol. (Reproduced by permission from *The Journal of Biological Chemistry*.[82])

should disappear, as it does in curve 3, and the 298 mμ band should increase, as in curve 4. This in itself is a reasonable identification, since it is based on a characteristic partition coefficient, a specific chemical reaction, and characteristic ultraviolet absorption. To determine whether an infrared analysis would give a suitable curve for identification purposes, it is only necessary to determine the purity of the material from an ultraviolet spectrum, which gives quantitative results more easily than an infrared spectrum. The findings for this case are shown in FIGURE 10. Curve *a* is the spectrum of the material from the peak quinone tube; curve *b* is this same material after reductive cyclization. The spectra characterize the quinone and phenol, respectively, and indicate that infrared analysis could yield a positive identification.

Separation of tocopherols and carotenes. This same isooctane-methanol system has been particularly useful for separating tocopherols and carotenes. A 24-plate distribution of a mixture of α-tocopherol and β-carotene gives complete resolution of the mixture. Tocopherol appears in tube 13 and the carotene in tube 22. The infrared spectra of each showed no presence of the other.

It is hoped that the preceding illustrations have demonstrated the potency of these fractionating procedures when they are combined with infrared anal-

ysis. It would appear that the utilization of several methods of purification would assure a greater usefulness of infrared spectrometry.

References

1. Blout, E. R. & H. Lenormant. 1955. Changes in the infrared spectra of solutions of deoxypentose nucleic acid in relation to structure. Biochim. et Biophys. Acta. **17:** 325.
2. Frick, G. & A. Rosenberg. 1954. Changes in the infrared absorption spectra of sodium desoxyribonucleate with *p*H and their interpretation on the basis of the Watson and Crick model. Biochim. et Biophys. Acta. **13:** 455.
3. Pollard, M., F. B. Engley, Jr., R. F. Redmond, H. I. Chinn & R. B. Mitchell. 1952. Infrared absorption spectra of viruses. Proc. Soc. Exptl. Biol. Med. **81:** 10.
4. Schwarz, H. P., R. Childs, L. Dreisbach & S. V. Mastrangelo. 1956. Quantitative infrared spectroscopy of desoxyribonucleic acid in the fractional milligram range. Science. **123:** 328.
5. Wilkins, M. H. F. & B. Battaglia. 1953. Note on the preparation of specimens of oriented sperm heads for X-ray diffraction and infrared absorption studies and on some pseudo-molecular behavior of sperm. Biochim. et Biophys. Acta. **11:** 412.
6. Blout, E. R. & M. Fields. 1950. Absorption spectra. VIII. The infrared spectra of some purines and pyrimidines. J. Am. Chem. Soc. **72:** 479.
7. Stimson, M. M. & M. J. O'Donnell. 1952. The infrared and ultraviolet absorption spectra of cytosine and isocytosine in the solid state. J. Am. Chem. Soc. **74:** 1805.
8. Willits, C. H., J. C. Decius, K. L. Dille & B. E. Christensen. 1955. Purines IV. The infrared spectrum of purine and certain substitued purine derivatives. J. Am. Chem. Soc. **77:** 2569.
9. Sinsheimer, R. L., R. L. Nutter & G. R. Hopkins. 1955. Infrared absorption spectra of pyrimidine nucleotides in H_2O and D_2O solution. Biochim. et Biophys. Acta. **18:** 13.
10. Tanner, E. M. 1956. Infrared absorption spectra of some hydroxypyrimidines. Spectrochim. Acta. **8:** 9.
11. Ambrose, E. J. & A. Elliott. 1951. Infrared spectroscopic studies of globular protein structure. Proc. Roy. Soc. **A208:** 75.
12. Elliott, A. & E. J. Ambrose. 1950. Evidence of chain folding in polypeptides and proteins. Discussions Faraday Soc. **9:** 246.
13. Chouteau, J. 1953. The interpretation of the infrared spectrum of proteins and the spectrographic modifications accompanying thermal denaturation. Compt. rend. **237:** 992.
14. Ehrlich, G. & G. B. B. M. Sutherland. 1953. Contribution of side chains to the infrared spectra of proteins: the 6.5 μ band. Nature. **172:** 671.
15. Freeman, N. K., F. T. Lindgren, Y. C. Ng & A. V. Nichols. 1953. Infrared spectra of some lipoproteins and related lipids. J. Biol. Chem. **203:** 293.
16. Goulden, J. D. S. 1956. Infrared absorption spectra and protein-sugar interactions. Nature. **177:** 85.
17. Lenormant, H. & E. R. Blout. 1953. Origin of the absorption band at 1550 $cm.^{-1}$ in proteins Nature. **172:** 770.
18. Morales, M. F., K. Lahi, J. Gergely & L. P. Cecchini. 1951. Some further infrared absorption studies on the proteins of muscle. J. Cellular Comp. Physiol. **37:** 477.
19. Parker, K. D. 1955. Infrared dichroism of fibrous proteins. Biochim. et Biophys. Acta. **17:** 148.
20. Blout, E. R. & A. Asadourian. 1956. Polypeptides. V. The infrared spectra of polypeptides derived from γ benzyl L glutamate. J. Am. Chem. Soc. **78:** 955.
21. Elliott, A. 1954. The infrared spectra of some optically active and mesosynthetic polypeptides. Proc. Roy. Soc. **A221:** 104.
22. Ellenbogen, E. 1956. Dissociation constants of peptides. III. The effect of optical configuration on the infrared spectra of polyfunctional peptides. J. Am. Chem. Soc. **78:** 366.
23. Brockmann, H. & H. Musso. 1956. Zur Kenntnis des IR Spektrums optisch aktiver Aminosäuren und ihrer Racemate. Chem. Ber. **89:** 241.
24. Davies, M. & J. C. Evans. 1953. The infrared absorptions of asparagine and glutamine. J. Chem. Soc. : 480.
25. Gäumann, T. & Hs. H. Günthard. 1952. Infrarotspektren von ω Aminosauren Helv. Chim. Acta. **35:** 53.
26. Koegel, R. J., J. P. Greenstein, M. Winitz, S. M. Birnbaum & R. A. McCallum.

1955. Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. V. Infrared spectra. J. Am. Chem. Soc. **77:** 5708.
27. Lenormant, H. 1946. The infrared absorption spectra of amino acids between 5 and 8 μ. J. chim. phys. **43:** 327.
28. Toribara, T. Y. & V. DiStefano. 1954. Infrared identification in paper chromatography applied to substances obtained from papergrams. Anal. Chem. **26:** 1519.
29. Wright, N. 1937. The infrared absorption spectra of the stereoisomers of cystine. J. Biol. Chem. **120:** 641.
30. Baer, E. 1953. On the crystallization, structure and infrared spectra of saturated L-α-lecithins. J. Am. Chem. Soc. **75:** 621.
31. Chapman, D. 1956. Infrared spectra and the polymorphism of glycerides. Part I. J. Chem. Soc. **:** 55.
32. Schwarz, H. P., R. Childs, L. Dreisbach & S. V. Mastrangelo. 1956. Infrared analysis of tissue lipids. Clin. Chem. **2:** 255.
33. Guertin, D. L., S. E. Wiberley & W. H. Bauer. 1956. Infrared absorption spectra of branched-chain fatty acids. Anal. Chem. **28:** 1194.
34. Herman, R. C. 1940. Vibration spectra and molecular structure. IX. Further studies of the vapors of the fatty acid series. J. Chem. Phys. **8:** 252.
35. Rigaux, C. 1954. Spectroscopie, modification des spectres d'absorption infrarouge des acides saturés aliphatiques qui accompagnent le passage de l'état liquide à l'état solide. Compt. rend. **238:** 63.
36. Barker, S. A., E. J. Bourne & D. H. Whiffen. 1956. Use of infrared analysis in the determination of carbohydrate structure. Methods of Biochem. Anal. **3:** 213. Interscience Publ. New York, N. Y.
37. Craven, C. W., K. R. Reissmann & H. I. Chinn. 1952. Infrared absorption spectra of porphyrins. Anal. Chem. **24:** 1214.
38. Rosenkrantz, H. 1948. Infrared absorption spectra of tocopherols and related structures. J. Biol. Chem. **173:** 439.
39. Rosenkrantz, H. & A. T. Milhorat. 1950. Some physical constants of α-tocopherylhydroquinone. J. Am. Chem. Soc. **72:** 3304.
40. Rosenkrantz, H. & A. T. Milhorat. 1950. Infrared absorption spectra of tocopherols and some of their chemical products. J. Biol. Chem. **187:** 83.
41. Rosenkrantz, H., A. T. Milhorat & M. Farber. 1952. Infrared absorption of sterols. II. Cholesterol and related sterols. J. Biol. Chem. **195:** 503.
42. Weigl, J. W. 1952. Infrared spectrum of deuterated ascorbic acid. Anal. Chem. **24:** 1482.
43. Anon. 1954. Spettro infrarosso del vitamina B_2 e pantotenato di calcio. Acta Vitaminol. **8:** 278, 279.
44. Anon. 1955. Spettro infrarosso del vitamina B_6 e piridoxamina. Acta Vitaminol. **9:** 77–79.
45. Rosenkrantz, H. 1955. The analysis of steroids by infrared spectrometry. *In* Methods of Biochemical Analysis. D. Glick, Ed. **2:** 1. Interscience Publ. New York, N. Y.
46. Jones, R. N. & C. Sandorfy. 1956. The application of infrared and Raman spectrometry to the elucidation of molecular structure. Technique of Organic Chemistry. **9:** 247. Interscience Publ. New York, N. Y.
47. Tswett, M. 1906. Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls. Ber. deut. botan. Ges. **24:** 384.
48. Diels, O. & H. F. Rickert. 1935. Über den Identitäts-Nachweis des Dehydrierungs-Kohlenwasserstoffes $C_{18}H_{16}$ aus Sterinen und Geninen mit γ-Methyl-cyclopentenophenanthren. Ber. deut. chem. Ges. **68:** 267.
49. Gordon, A. H., A. J. P. Martin & R. L. M. Synge. 1944. Technical notes on the partition chromatography of acetamino acids with silica gel. Biochem. J. **38:** 65.
50. Silberman, H. & S. Silberman Martynowa. 1940. The chromatographic separation of bile acids. I. The separation of cholic from desoxycholic acid. J. Biol. Chem. **165:** 359.
51. Lieberman, S., K. Dobriner, B. R. Hill, L. F. Fieser & C. P. Rhoads. 1948. Studies in steroid metabolism. II. Identification and characterization of ketosteroids isolated from urine of healthy and diseased persons. J. Biol. Chem. **172:** 263.
52. Boscott, R. J. 1947. Solvent-treated cellulose acetate as the stationary phase in partition chromatography. Nature. **159:** 342.
53. Dirscherl, W., W. Korfs & H. Schriefers. 1956. Trennung von Steroiden durch Verteilungschromatographie an Cellulosesäulen. Hoppe Seyler's Z. physiol. Chem. **305:** 116.

54. Briggs, S. W., R. G. Denkewalter & G. B. Hughey. 1954. Vitamin B_{12} elution from charcoal. Chem. Abstr. **48:** P10306*i*.
55. Moore, S. & W. H. Stein. 1949. Chromatography of amino acids on starch columns. Solvent mixtures for the fractionation of protein hydrolysates. J. Biol. Chem. **178:** 53.
56. Butt, W. R., P. Morris, C. J. O. R. Morris & D. C. Williams. 1951. The polarographic estimation of steroid hormones. V. Determination of progesterone in blood. Biochem. J. **49:** 434.
57. Howard, G. A. & A. J. P. Martin. 1950. The separation of the C_{12}-C_{18} fatty acids by reversed-phase partition chromatography. Biochem. J. **46:** 532.
58. Nyc, J. F., D. M. Mawn, J. B. Garst & H. B. Friedgood. 1951. Chromatographic separation of estrone, estradiol and estriol. Proc. Soc. Exptl. Biol. Med. **77:** 466.
59. Reichstein, T. 1936. Über Bestandteile der Nebennierenrinde. VI. Trennungsmethoden, sowie Isolierung der Substanzen Fa, H und J. Helv. chim. Acta. **19:** 1107.
60. Martin, A. J. P. & R. L. M. Synge. 1941. A new form of chromatogram employing two liquid phases. I. A theory of chromatography. Biochem. J. **35:** 1358.
61. Alm, R. S., R. J. P. Williams & A. Tiselius. 1952. Gradient elution analysis. I. A general treatment. Acta Chem. Scand. **6:** 826.
62. Lakshmanan, T. K. & S. Lieberman. 1954. An improved method of gradient elution chromatography and its application to the separation of urinary ketosteroids. Arch. Biochem. and Biophys. **53:** 258.
63. Partridge, S. M. & R. G. Westall. 1949. Displacement chromatography on synthetic ion-exchange resins. I. Separation of organic bases and amino acids using cation-exchange resins. Biochem. J. **44:** 418.
64. Reichberg, D. 1956. The use of ion-exchange resins in partition chromatography. Chem. & Ind. **:** 958.
65. Consden, R., A. H. Gordon & A. J. P. Martin. 1944. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. J. **38:** 224.
66. Touchstone, J. C. & C. Hsu. 1955. Determination of α-ketolic substances in urinary extracts and paper chromatograms. Anal. Chem. **27:** 1517.
67. Burton, R. B., A. Zaffaroni & E. H. Keutmann. 1951. Paper chromatography of steroids. II. Corticosteroids and related compounds. J. Biol. Chem. **188:** 763.
68. Bush, I. E. 1952. Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J. **50:** 370.
69. Calvin, M. 1956. The photosynthetic cycle. J. Chem. Soc. **:** 1895.
70. Kalkwarf, D. R. & A. A. Frost. 1954. Use of infrared radiation for detection of colorless substances on paper chromatograms. Anal. Chem. **26:** 191.
71. Kritchevsky, D. & M. Calvin. 1950. Paper chromatography of steroids. J. Am. Chem. Soc. **72:** 4330.
72. Brown, F. 1952. The estimation of vitamin E. I. Separation of tocopherol mixtures occurring in natural products by paper chromatography. Biochem. J. **51:** 237.
73. Kritchevsky, T. H. & A. Tiselius. 1951. Reversed phase partition chromatography of steroids on silicone-treated paper. Science. **114:** 299.
74. Haugaard, G. & T. D. Kroner. 1948. Partition chromatography of amino acids with applied voltage. J. Am. Chem. Soc. **70:** 2135.
75. Voigt, K. D. & I. Beckmann. 1953. Paper electrophoresis of steroids. Acta Endocrinol. **13:** 19.
76. Craig, L. C. 1944. Identification of small amounts of organic compounds by distribution studies. II. Separation by counter-current distribution. J. Biol. Chem. **155:** 519.
77. Perry, E. S. & W. H. Weber. 1954. Automatic drive for counter-current distribution apparatus. Anal. Chem. **26:** 498.
78. Williamson, B. & L. C. Craig. 1947. Distribution studies. Calculation of theoretical curves. J. Biol. Chem. **168:** 687.
79. Craig, L. C., G. H. Hogeboom, F. H. Carpenter & V. du Vigneaud. 1947. Separation and characterization of some penicillins by the method of counter-current distribution. J. Biol. Chem. **168:** 665.
80. Livermore, A. H. & V. duVigneaud. 1949. Preparation of high potency oxytocic material by use of counter-current distribution. J. Biol. Chem. **180:** 365.
81. Engel, L. L., W. R. Slaunwhite, Jr., P. Carter & I. T. Nathanson. 1950. The separation of natural estrogens by counter current distribution. J. Biol. Chem. **185:** 255.
82. Rosenkrantz, H., A. T. Milhorat & M. Farber. 1951. Counter-current distribution in identification of tocopherol compounds in feces. J. Biol. Chem. **192:** 9.

83. Forchielli, E., H. Rosenkrantz & R. I. Dorfman. 1955. Metabolism of 11-deoxycortisol *in vitro*. J. Biol. Chem. **215:** 713.
84. Savard, K., S. Burstein, H. Rosenkrantz & R. I. Dorfman. 1953. The metabolism of adrenosterone *in vivo*. J. Biol. Chem. **202:** 717.
85. Hagopian, M., G. Pincus, J. Carlo & E. B. Romanoff. 1956. Isolation of an unknown substance and 6-ketoprogesterone from perfusates of human placentae. Endocrinology. **58:** 387.
86. Burstein, S. & R. I. Dorfman. 1955. Steroid metabolism in guinea pigs. I. Metabolism of cortisol *in vivo*. J. Biol. Chem. **213:** 581.
87. Rosenkrantz, H. & R. I. Dorfman. 1953. The *in vitro* metabolism of dehydroepiandrosterone. Abstr. 19th Intern. Physiol. Congr. Montreal. : 713.
88. Rosenkrantz, H. 1953. An antimony trichloride reagent suitable for the detection and estimation of nonketonic steroids. Arch. Biochem. Biophys. **44:** 1.
89. Denison, F. W. & E. F. Phares. 1952. Rapid method for paper chromatography of organic acids. Anal. Chem. **24:** 1628.
90. Rosenkrantz, H., A. T. Milhorat, M. Farber & A. E. Milman. 1951. Purification and identification of the antistiffness factor. Proc. Soc. Exptl. Biol. Med. **76:** 408.
91. Milhorat, A. T., J. B. Mackenzie, S. Ulick, H. Rosenkrantz & W. E. Bartels. 1949. Observations on a biologically active vitamin E derivative present in hog gastric mucin and in hog stomach lining. The biologic activity of dl, alpha-tocopherylhydroquinone. Ann. N. Y. Acad. Sci. **52**(3): 334.
92. Butt, W. R., A. A. Henly, & C. J. O. R. Morris. 1948. The polarographic estimation of steroid hormones. IV. Determination 3α- and 3β-hydroxy-17-ketosteroids. Biochem. J. **42:** 447.

AUTOMATIC ANALYSIS OF INFRARED SPECTRA

By Mortimer Rogoff
Federal Telecommunication Laboratories, Nutley, N. J.

I shall discuss a group of related projects currently in progress at Federal Telecommunication Laboratories. Since we are primarily electronic engineers, it may seem strange for me to report on work that is aimed directly at infrared spectroscopic analysis. However, what we are trying to do is to apply to specific problems in the analysis of infrared spectra the methods of rapid-computing techniques that have evolved in the field of electronics. One of the major contributions of electrical engineering in the past few years has been the solution of arithmetic problems by high-speed electronic techniques. Problems that could be handled in minutes or seconds on conventional electrical hand calculating machines can now be solved in microseconds or milliseconds by using vacuum tubes and transistors packaged in large but silent cabinets. The advent of these machines has released people from hours or weeks of routine calculations. In these days, when ideas come faster than they can be exploited, it is essential that we provide some means of freeing our thinking people from this drudgery so that their concepts may be developed into useful contributions to science.

Clearly, another important aspect is the capacity to do a given job with greater thoroughness and more intrinsic accuracy. The high-capacity, high-speed electronic calculator will do the same computation over and over again, examining small shifts in any of the physical quantities to determine whether the correct solution has been made. The machine does not mind doing this work over and over again, and it can examine small and subtle manipulations made to its input data in order to be sure that the problem at hand is being solved with completeness.

In the field of infrared analysis we have not seen a relatively simple electronic calculator that can be applied to routine or research problems. A general-purpose computing facility is not always the wisest choice for a specific field of work; a calculator capable of handling computing problems in such diverse fields as general accounting, inventory accounting, information retrieval, and sales analysis is not necessarily either the cheapest, smallest, or fastest machine to do a specific problem. In our work in spectroanalysis we have evolved methods of computation that involve fairly simple arithmetic and a limited number of program instructions for the machine. It is our feeling that a special-purpose machine can be made that will handle a large variety of problems in infrared analysis and yet be substantially smaller and cheaper than some of the general-purpose models. For this reason we have felt it advisable to make a substantial effort to produce yet another electronic calculating machine that we think will have wide application in the specific field of chemical analysis.

Let us now turn to some of the specific work that we are doing in this field. At first we hoped to be able to analyze the spectrogram of a mixture for both the identity and amount of each of its constituents. We expected to perform

this analysis by comparing the unknown spectrogram to combinations of selected reference spectra. After a period of mathematical investigation it became apparent that a given "mixture spectrogram" could be described as the linear combination of constituent absorption spectrograms, but that a knowledge of the identity of each of the constituents in the unknown was required. If this requirement was fulfilled, then it was possible to form a synthetic "mixture spectrogram" by combining appropriate amounts of the reference spectra to match the unknown. We approached this entire problem as one of wave-form analysis, which is a field of interest to all communication engineers. It was hoped that apparatus that we have used in the past in the field of communications could be applied to the analysis of infrared spectrograms.

I should like to emphasize that all our thinking is based on the manipulation of infrared absorption spectra themselves; we regard them as the fundamental entity. We do not probe into the phenomenon of molecular absorption; we start from the "fingerprint" spectrogram obtained from a spectrophotometer.

I have mentioned the requirement of prior qualitative analysis. Such an analysis may not always be available in advance of the solution of a given problem; however, as we apply our methods to specific problems, we find that this becomes a less severe restriction. Usually, a specific analytical situation is a bound problem; it does not involve all possible chemical constituents. The analyst, by his own knowledge of the possible constituents, can reduce the number of reference spectra that he must use. I shall return to this initial requirement for qualitative analysis later.

If we assume that we have available a library of recorded spectra of pure constituents that contains all the members of a given unknown, then our next problem is to combine these spectra in the proper amounts so that we may match the unknown. The inclusion of constituents other than those actually present in the unknown causes no difficulty. Our analysis will show these extra members to be of zero concentration, and this result will be displayed in the final answer.

In FIGURE 1 is illustrated a simple model of an automatic calculator that will solve this problem of quantitative analysis in a direct and straightforward way. The mixture spectrogram shown is obtained by adding together the absorption spectra of four constituents, each taken in an amount given by C_1 through C_4 inclusive. Obviously, the correct solution to this problem is obtained when the matching coefficients C'_1 through C'_4 are equal to C_1 through C_4. However, we start our problem without knowledge of the relative amounts of each of the four constituents. We take arbitrary amounts of the library constituents and combine them to produce what we call a synthesized spectrum. If we subtract the mixture spectrogram from this synthesized spectrogram we obtain another spectrum called the error spectrum. This spectrogram can possess both positive and negative values of absorption because of the arbitrary settings of the concentration controls used to obtain the synthetic mixture.

There are two ways to manipulate the concentration controls of the synthetic spectrogram in order to obtain a perfect match to the unknown. The first of these is "hunt and peck." If we are observing the error spectrogram (say, on

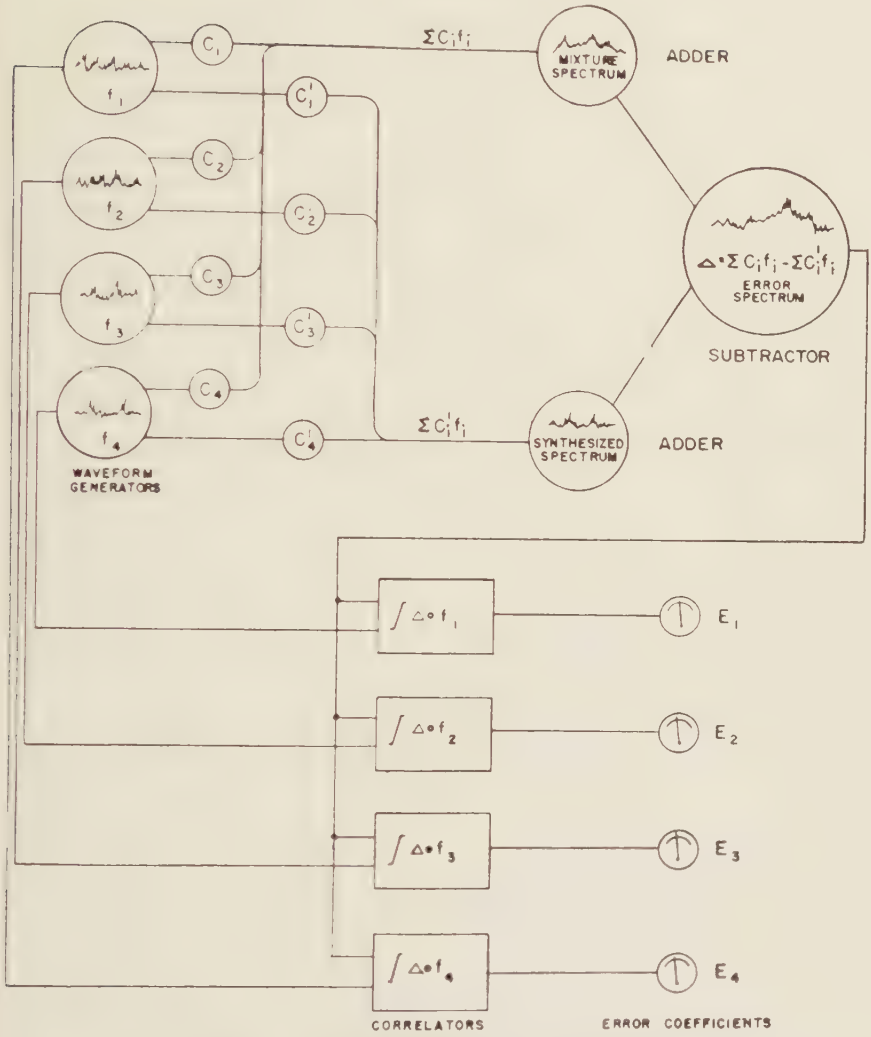


FIGURE 1. Infrared-spectrum computing analyzer.

an oscilloscope) we can arbitrarily adjust each of the library coefficient controls until we observe that the error spectrogram has become a zero-valued quantity at all wave lengths; but this is hardly an automatic procedure. Fortunately, there is a direct way of adjusting these synthetic spectrogram constituents that can be developed into an automatic process: this is the process of correlation shown in the illustration. Speaking broadly, what we shall do is to make zero the correlation between the error spectrum and each of the constituents that, because of wrong amounts, have created this error spectrogram. These correlators are electronic devices that produce the integral of the product of the error spectrum and each constituent of the synthetic spectrum. When each

of these correlation coefficients is separately made to be zero by adjusting each of the concentration controls of the members of the synthetic spectrum, the best match to the unknown spectrogram will have been made.

In the special case that we have depicted in this illustration, where the best match is a perfect match, it is clear that all of the correlation integrals will be made zero because the common factor in each of these multipliers (the error spectrum) will have become zero-valued at the time that the match is made. Therefore, all of the error coefficients will be simultaneously zero because all of the integrals will be zero. The important characteristic of this device is the behavior of an error coefficient as its corresponding constituent control is manipulated. If f_1 is in too large an amount in the synthetic spectrum, because C_1' allows too much of this constituent to be present in the synthetic spectrum, then E_1 will indicate a quantity larger than zero. By observing the value of E_1, C_1' can be readjusted to bring E_1 to zero. One need not look at the behavior of the error spectrum while this adjustment is being made. Thus, it is a simple matter to close the loop between E_1 and C_1' by means of a servomechanism in order to adjust C_1' automatically to make E_1 zero at all times. With a servomechanism connecting each of the error coefficients to its appropriate concentration control, the whole problem will be solved quickly and automatically. In the design of the servomechanisms of the system, allowance must be made for interaction among these controls, due to the overlap of absorption bands in the constituent spectra. But there is only one true answer, and this will be the equilibrium solution obtained after all the servomechanisms have adjusted their appropriate controls to yield zero-value error coefficients.

Probably the most important feature of our method is that we make a match on the basis of a whole absorption spectrogram. You will notice that we have not made any selection of particular wave lengths to describe combinations of spectra. We are matching spectra on the basis of their entire shape in order to obtain a match that is equally good across the entire interval of wave length under consideration. By this means, utilizing the smoothing action of integral analysis, we can reduce a number of errors that can appear in the usual point-by-point methods. For example, the effects of residual instrument noise are well attenuated by this method.

Matching of spectra over a continuous interval of wave length enables us to perform an analysis in any interval that contains the unique fingerprint region. If, in this region, there is interaction and nonlinear combination of constituent spectra, we can materially reduce errors that might be created by such behavior merely by blanking out the offending portions of the interval. If the analyst has reason to expect substantial nonlinearity in a region or regions of the spectrogram being analyzed, he can eliminate these regions from the computation and perform the analysis with the remaining sections. We have experimented with this procedure and have found a noticeable improvement in accuracy of the result. This approach is one way to eliminate the difficulties inherent in linear analysis when nonlinear behavior is present. Sufficient information remains to provide an accurate analysis.

Another important feature is the fact that an over-all check on the quality of the result is available by observing the error spectrogram that remains after

the computation is complete; one is able immediately to judge the quality of the result. For example, if a wrong choice of constituent spectra has been made in producing the synthetic spectrogram (because of incorrect qualitative analysis preceding the computation) an interesting phenomenon results. The method of computation inherently produces a set of zero-valued error coefficients, even if one reference spectrum is not correct, because this method is merely one of least-square error manipulation: we are finding that synthetic spectrogram which makes the best match to the unknown at all wave lengths on a least-squared error basis. Any set of arbitrary spectra will make a match to an unknown on this basis because we have only to minimize the squared error to fit our criteria. Therefore we need some way of determining whether the obtained solution is proper. This we can deduce by observing the error spectrogram. If it is zero-valued for all wave lengths at the end of the computation, then we can be assured that the least-squared error match is the proper match and that these are the answers for which we have been searching. If, on the other hand, the least-squared error match yields a finite error spectrogram, then our choice of reference spectra is wrong. This is an important asset to the analyst in two ways: first, he is informed that his choice of match constituents is improper; second, when he observes the error spectrogram he is given some clue as to the reason for the lack of fit. In some cases the error spectrogram will possess an absorption band or bands that immediately point the way to the proper choice of constituent to obtain the correct solution. This is a factor that should be of importance when difficult problems of analysis are encountered.

The machines under construction at the Federal Telecommunication Laboratories are substantially different in appearance from the one illustrated here, although their method of solution is identical. Instead of using what we call analogue methods, whereby spectra are treated much as they are produced on a spectrophotometer, we shall immediately convert these spectrograms to digital quantities. The spectrogram will be converted to a digital record of absorbance, and all the necessary arithmetic will be treated in digital fashion. This will permit greater accuracy than can be obtained by analogue methods.

I should like to describe some work that we have done in association with the Sloan-Kettering Institute for Cancer Research in New York. We decided to apply our method as soon as possible to actual problems in biochemistry to ascertain whether it could be of material assistance in an actual analytical problem. The staff of the Sloan-Kettering Institute generously cooperated by supplying us with spectra obtained from an actual problem. These spectra are illustrated in FIGURES 2 to 9. We analyzed a natural mixture of two steroids obtained from a fraction of urine by comparison with spectra of the pure compounds contained in the mixture. In addition to the named constituents, there is in the mixture spectrogram a background that is of little interest for the analysis. However, we cannot ignore this background in our method, for it is in fact a third constituent that must be handled in the same fashion as any other member of the unknown. The spectrum of this background material was obtained by producing a differential spectrogram between the natural mixture and a mixture of the two desired steroid constituents in a double-beam

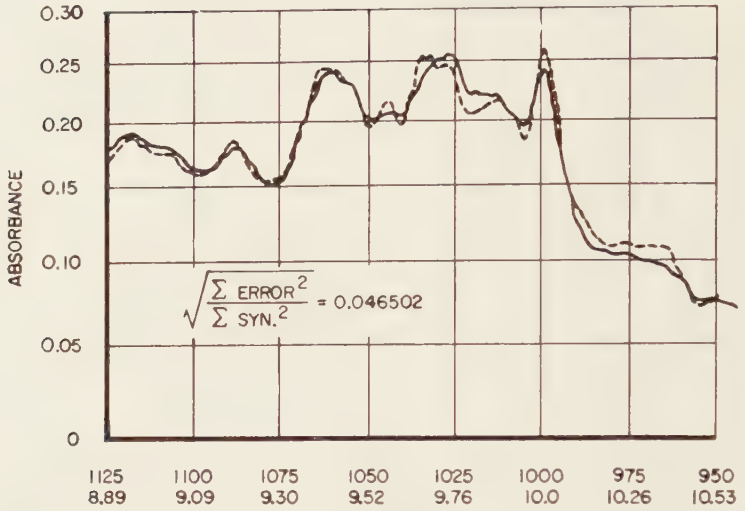


FIGURE 2. Sample No. 4: ——— natural mixture; – – – – computed synthesis. C_1 = 0.571953, androsterone; C_2 = 0.377951, etiocholanolone; C_3 = 0.859938, background.

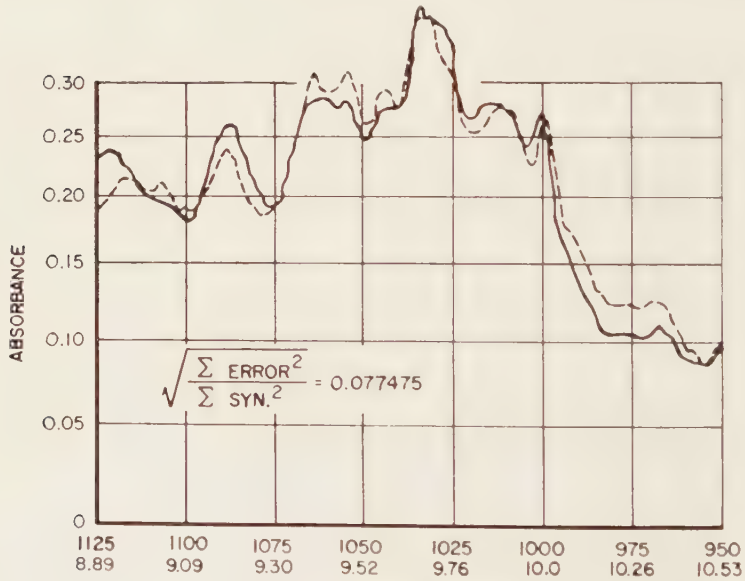


FIGURE 3. Sample No. 5: ——— natural mixture; – – – – computed synthesis. C_1 = 0.492333, androsterone; C_2 = 0.663402, etiocholanolone; C_3 = 0.936875, background.

spectrophotometer. The relative amounts of the two pure steroids were adjusted until there was no recognizable presence of steroid material in the differential spectrogram. The result was the spectrum of the background. Once obtained, this spectrogram was used as the third constituent in our library of

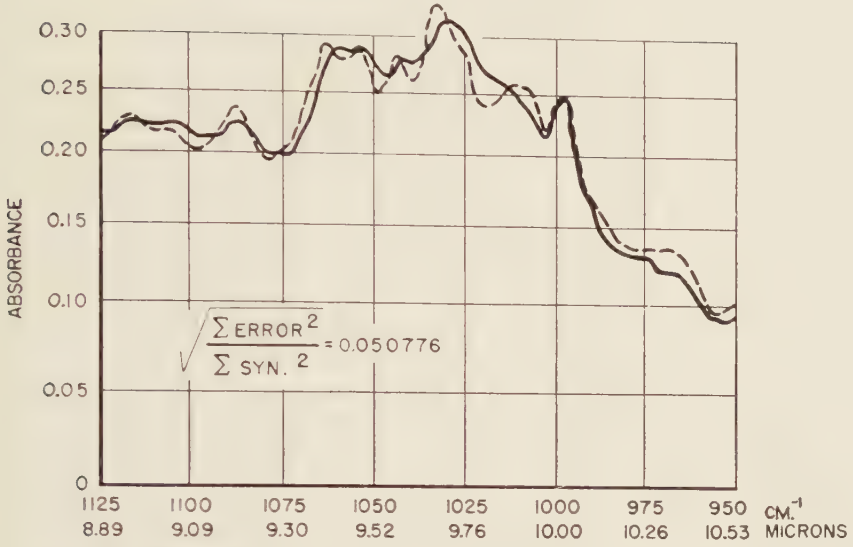


FIGURE 4. Sample No. 6: ——— natural mixture; – – – – computed synthesis. C_1 = 0.448589, androsterone; C_2 = 0.515791, etiocholanolone; C_3 = 1.168620, background.

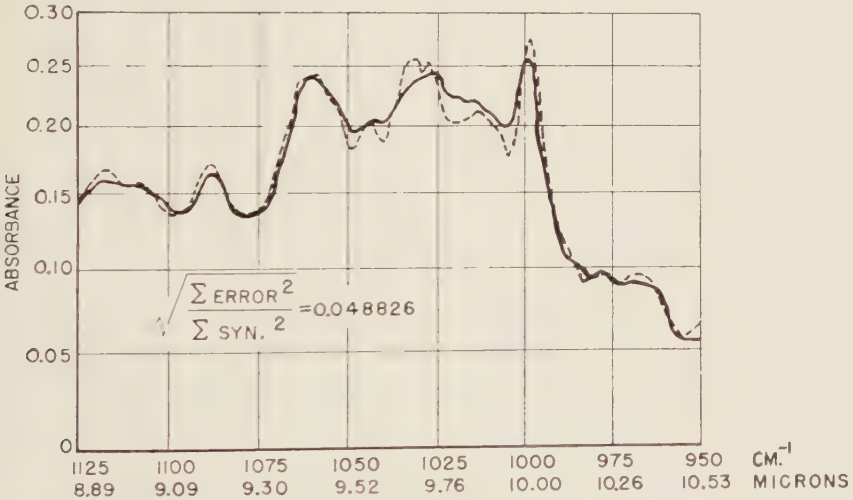


FIGURE 5. Sample No. 7: ——— natural mixture; – – – – computed synthesis. C_1 = 0.644514, androsterone; C_2 = 0.412143, etiocholanolone; C_3 = 0.555639, background.

reference spectra. The computation was carried out and numerical results were obtained. These concentration coefficients were then applied to the library spectra and the appropriate amounts of each of the three spectra were combined to produce a synthetic mixture spectrogram. This has been plotted on the same charts as the original mixture and a visual comparison can be made as to the quality of the result. For each of the solutions we have computed a

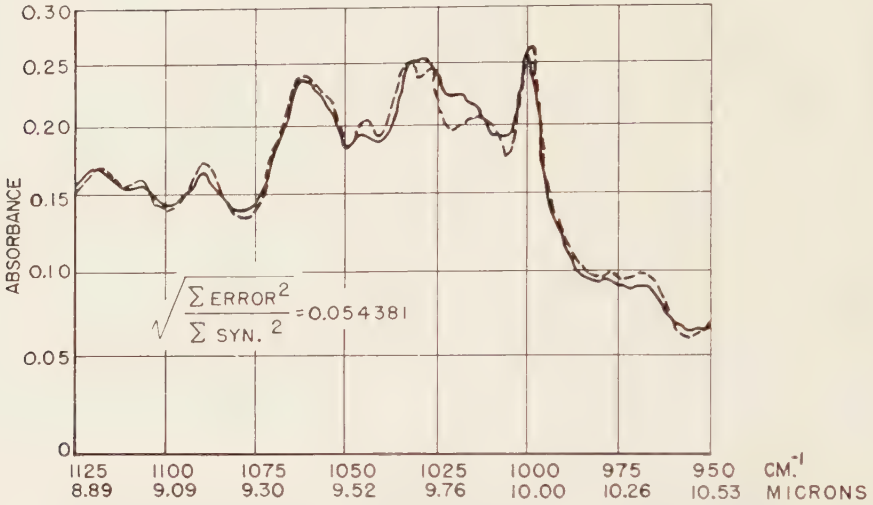


FIGURE 6. Sample No. 8: ——— natural mixture; – – – – computed synthesis. C_1 = 0.599719, androsterone; C_2 = 0.412143, etiocholanolone; C_3 = 0.555639, background.

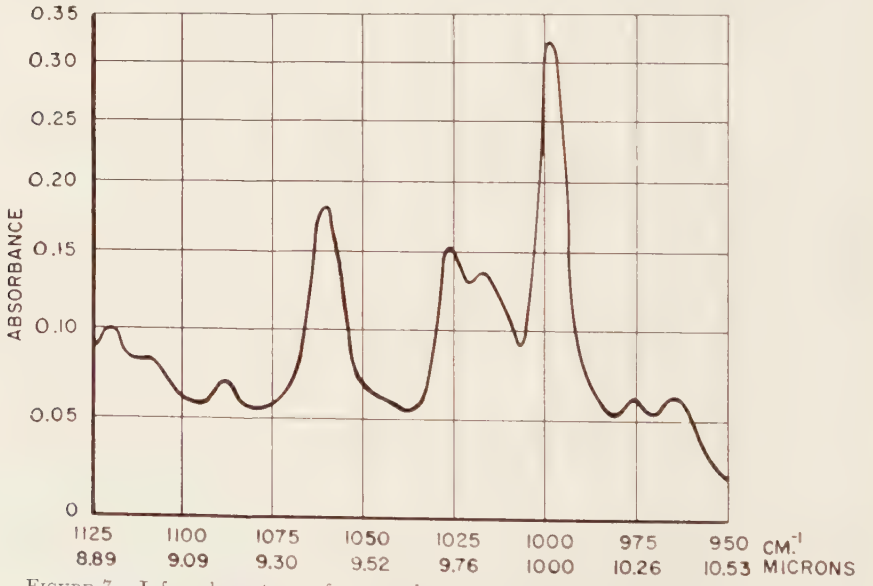


FIGURE 7. Infrared spectrum of pure androsterone, 1.03 mg. in 397 mg. $CHCl_3$.

"quality" number, which is merely the ratio of the squared error in the set to the squared area under one of the two curves. These results are well within the experimental errors involved.

These charts are shown in FIGURES 2 to 5. Inspection of the superimposed curves indicates the quality of match between the computed synthesis and the

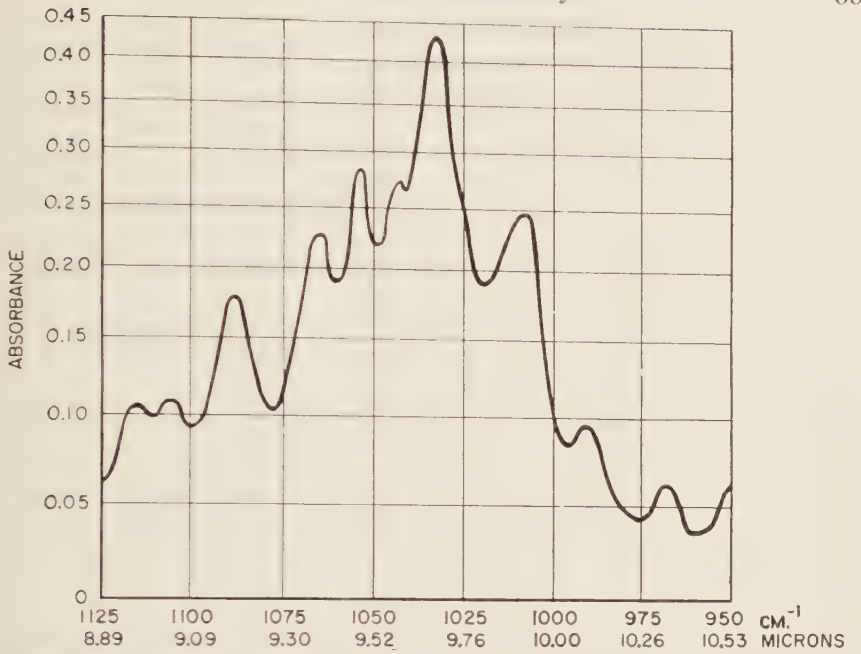


FIGURE 8. Infrared spectrum of pure etiocholanolone, 1.22 mg. in 316 mg. $CHCl_3$.

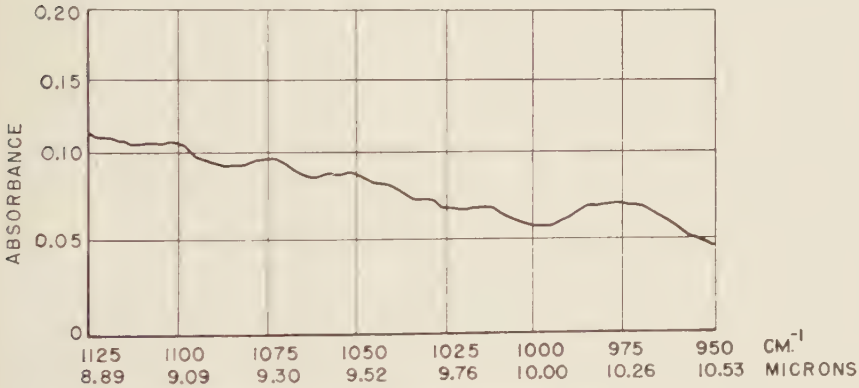


FIGURE 9. Differential background spectrogram.

natural mixture in each case. Also shown in each chart is the number that describes the quality of the fit; these vary from 4.6 to 7.7 per cent. The reference spectra from which the synthesis was completed are shown in FIGURES 7 to 9. The differential background spectrogram of FIGURE 9 was applied in all five cases of computation.

It may be stressed that the background spectrogram determined by differential analysis from one natural mixture was used for all five unknowns obtained from five different patients. It is interesting that this one background could

be used in all these problems and still yield results of useful accuracy. In one sense, this is an answer to the requirement of prior qualitative analysis. If we have little interest in a whole group of constituents in an unknown, and if this group as a whole has a constant behavior as far as its spectrum is concerned, then it can be considered merely as one additional unknown. If we can once determine the spectrogram of this entire group, and use it as a constant reference, we can "see through it" to the remaining constituents of interest. It is undoubtedly true that much of the nature of the background material in this problem is the result of the particular method of chemical fractionation preceding our analysis. This would explain to some extent the constancy of the results when the single background was applied to all mixtures. Other variability from unknown to unknown is probably attributable to shifts in the composition of this background material.

It is possible that a library of background spectra could be produced in order to encompass all forms of variation that would normally be encountered in routine analysis. If we have available rapid computing machines, we can try many solutions, changing the background choice each time until we reach the best solution, as evidenced by the quality of the fit. This is a valuable application of a rapid computer, since it allows accurate analytical answers even though we are not entirely sure of some members of the unknown mixture.

We are continuing our study of this type of problem by expanding the number of unknowns handled in the computation. At the present time we have not yet completed a problem containing eight constituents. We expect these results shortly, and we look forward to accuracies similar to those already obtained.

It should be clear that an important part of the computing facility is access to a library of recorded spectra. The whole method is based upon combination of recordings of pure constituents in order to simulate the unknown. The power of the method will depend upon the scope of the library available to the user. Toward this end we have under way at our laboratories a project the purpose of which is to produce recordings of spectra in a compact digital form, capable of extremely rapid readout into the computing equipment. We propose to use photographic film as our storage medium. Our goal is to record digits of absorbance in the interval of 650 to 5000 cm. $^{-1}$, all within one inch of 35 mm. film. We believe that we can read this back into the computer in approximately one tenth of a second. With this type of storage system it is conceivable to have readily available many thousands of spectra for comparison and computational processes.

When computing machines of the type that we have described are initially in use it may not be necessary to have a vast library of reference spectra. Only a few problems will be applied, requiring a reference library of reduced scope. We shall probably use punched paper tape or magnetic tape recording facilities initially. As the need grows, we can convert this to a more compact form of film storage. We see this type of film library as an ultimate goal as we progress toward more and more machine computation in this field.

One other field of work that we have begun in our laboratories will have, if it succeeds, direct bearing on infrared absorption analysis. In our physical

chemistry laboratory, which has been intensely active in semi-conductor research in the last decade, new forms of infrared detectors are currently being investigated. Our goal is to produce a detector of this type with useful response to 650 cm. [1] If we can achieve the necessary sensitivity and low noise output, we shall have an infrared detector with time constant within the order of microseconds. This is perhaps one or two orders of magnitude more rapid than is obtained from the thermal detectors used today. With such performance we shall be able to produce infrared absorption spectrograms in a matter of seconds rather than in the minutes now required. Such speed goes hand in hand with the speed inherent in the electronic computation that will be applied to analysis of these spectrograms.

If we can bring speed and accuracy into the handling of analytical problems in this branch of chemistry, we think that we can materially assist the analyst in more effective use of the infrared technique. This increased ability to handle complicated mixtures will reduce the necessity for laborious physical and chemical separation before quantitative results are obtained. We think that this speed will allow more productive man-hours in the laboratory and thereby hasten the solution of complex problems.

Acknowledgment

I should like to acknowledge fundamental assistance given to me by certain individuals within our organization. This whole project stems from original work contributed by Henri Busignies, President of Federal Telecommunication Laboratories. It was his concept to apply electronic techniques to the special field of medical technology. George Deschamps contributed the mathematical background upon which we have based the design of our computers. Paul Lighty has given material assistance in directing our work by sharing with us his experience in the field of applied spectroscopy. In addition, his department is responsible for the work in improved infrared detectors.

INFRARED INTENSITY MEASUREMENTS APPLIED TO THE DETERMINATION OF MOLECULAR STRUCTURE*

By R. Norman Jones, E. Augdahl†, A. Nickon†, G. Roberts†, and D. J. Whittingham†

Division of Pure Chemistry, National Research Council of Canada, Ottawa, Canada

Introduction

Chemists have been using absorption spectra for many years as aids to the interpretation of the molecular structure of complex organic compounds. The spectra studied in the ultraviolet and visible regions are almost invariably reported on an absolute intensity basis of molecular extinction coefficient ($\epsilon_{max.}$). The $\epsilon_{max.}$ values are a useful means of distinguishing between different ultraviolet chromophores that absorb in the same region of the spectrum, and they are also useful for evaluating purity, particularly in following the isolation of pure compounds from natural sources.

A few infrared spectroscopists, including Rose,[1] Anderson and Seyfried,[2] Hampton and Newell,[3] McMurry and Thornton,[4] Cross and Rolfe,[5] and Reggiani, Casu, and Caroti,[6] have used molecular extinction coefficients, but it is still the general practice to express infrared band intensities in arbitrary units of percentage absorbed or percentage transmitted. For spectra measured in the solid phase as Nujol mulls or halide disks, the extinction coefficients cannot be determined, as there are no adequate methods to correct for scattering errors or for the nonhomogeneous distribution of the sample. However, for spectra measured in solution the molecular extinction coefficients can be evaluated in the same way as in the visible and ultraviolet regions of the spectrum.

It has been recognized for a long time that measurements of infrared intensity are liable to greater errors than similar measurements made at higher frequencies, and it is often stated that infrared molecular extinction coefficients are subject to such large errors that they cannot usefully be transferred from one spectrometer to another. This is a considerable exaggeration. It is true that there are instrumental factors that make it advisable, for the present, to report infrared intensities in "apparent" units; nevertheless, if proper precautions are taken in setting up and operating the spectrometer these errors need not be excessive.

Our laboratory has been looking into this problem with respect to the measurement of both molecular extinction coefficients and integrated absorption intensities, and the reproducibility of such measurements on various types of spectrometers has been examined. The conclusions of this study will be mentioned briefly below and described in more detail in a forthcoming publication.[7] It should be emphasized at the outset that with the instruments now available, the recording of absolute intensity measurements is slow and

* Published as Contribution No. 4373 from the Laboratories of the National Research Council of Canada.

† National Research Council of Canada Post-doctorate Fellow.

laborious, particularly for investigators who are conditioned to the rapid presentation of qualitative spectra on fast recording double-beam spectrophotometers.

We have now been working with infrared spectra plotted as extinction coefficient ϵ against wave number* ν for a sufficient length of time to visualize the spectra directly in these terms, and in our experience the effort involved is justified by the clearer concept this method provides of the relative significance of the various types of group frequency vibrations in their over-all effect on the band envelope. This makes it possible to predict more effectively what will be the consequences of small changes in molecular structure, and it also provides a better insight into the nature of the vibrations in the complex molecule.

The absolute accuracy of such intensity measurements cannot be evaluated at present, as there are no standards for comparison purposes. There is urgent need that such standards be established on the basis of carefully regulated measurements, preferably using several high-resolution grating spectrometers in a number of different laboratories.

The principles involved in making infrared intensity measurements will be discussed first, and illustrated later by applications to specific problems. The use of integrated absorption intensities and molecular extinction coefficients will be dealt with separately.

Measurement of Infrared Band Intensities

The term *extinction coefficient* for the measure of absorption intensity was first introduced by Bunsen and Roscoe[3] in 1861, and the molecular extinction coefficient has been used for a quarter of a century to measure the intensities of absorption bands in the ultraviolet and visible. It is convenient to use the same term for infrared absorption bands, and ϵ_ν, the molecular extinction coefficient at the wave number ν, can be defined as

$$\epsilon_\nu = \frac{1}{cl} \cdot \log_{10}\left(\frac{I_o}{I}\right)_\nu \tag{1}$$

where c is the concentration of the solute in moles per liter of solution, l the cell length in cm., and $\log_{10}(I_o/I)_\nu$ the absorbance (optical density) of the solution. It is assumed that the solvent is nonabsorbing, or else that its absorption is compensated. For dilute solutions compensation can be made with a matched cell containing the solvent only, but if the solvent is appreciably absorbing a correction may be necessary for the mole fraction of the solute at high concentrations (Jones and Sandorfy,[4] p. 265). The common group frequency vibrations are associated with $\epsilon_{max.}$ values in the range 1 to 1000.

Theoretically, $\epsilon_{max.}$ cannot be directly related to the electrical character of the vibration, but the total area under the absorption band, the *integrated*

* *Consulting Editor's note*: In the present paper the symbol ν represents the frequency of the radiation divided by the speed of light; that is, the wave number. This quantity is represented by $\tilde{\nu}$ elsewhere in this monograph.

absorption intensity, A, depends on the change of the electric moment during the vibration. This aspect of infrared intensity measurement has been dealt with in a recent review by Thompson.[10]

Physicists commonly define the integrated absorption intensity as

$$A = \frac{1}{cl} \int \log_e \left(\frac{I_o}{I} \right)_\nu d\nu \tag{2}$$

$$= 2.303 \int \epsilon \, d\nu \tag{3}$$

The integration theoretically extends over the whole band, but in practice the limits are determined by overlap with neighboring bands, and some kind of empirical correction must be added for the "wings" beyond the practical limits of measurement.

The choice of units for A is in a confused state. Some investigators introduce the Avogadro number N into the denominator of EQUATION 2 to express the absorption on a molecular rather than a molar basis. Others introduce the speed of light to convert from wave number, in cm.$^{-1}$, to absolute frequency, in cycles per second. For most chemical purposes the unit as defined by EQUATION 3 is satisfactory. For the stronger bands it takes on values in the range 1×10^4 to 5×10^4 mole^{-1} liter cm.$^{-2}$, and it is convenient to define 10^4 mole^{-1} liter cm.$^{-2}$ as *one unit of integrated absorption intensity*.

It can be argued that the exponential factor of 2.303 could well be dropped from EQUATION 3 and A redefined as

$$A = \int \epsilon_\nu \, d\nu \tag{4}$$

but we hesitate to make any further change pending the establishment of some general convention.

For many purposes it is also necessary to have some acceptable measure of the band width, and for this the *half intensity band width* ($\Delta\nu_{1/2}$) can be used. This is the width of the band in cm.$^{-1}$ where $\epsilon = 0.5\ \epsilon_{max}$.

Errors due to the finite slit width. The quantities defined involve $(I_o, I)_\nu$, which assumes that the radiation is monochromatic. Unfortunately, the energy passing through the exit slit of a spectrometer illuminated with continuous radiation is never strictly monochromatic, and the departure from monochromaticity is determined by the spectral slit width, S, measured in cm.$^{-1}$

For prism spectrometers with Littrow mounting, S is given by a complex expression of the form

$$S = \Sigma + F(s)\Pi + \Phi \tag{5}$$

where Σ and Π are functions of the optics and geometry of the spectrometer (Jones and Sandorfy,[9] p. 274; Williams[11]). $F(s)$ is a proportionality factor and Φ a correction for such factors as aberration and misalignment of optics.

Unfortunately, S cannot be computed directly because there is uncertainty in the selection of the proper values for $F(s)$ and Φ. If we arbitrarily take $F(s)$ as unity and Φ as zero, the errors introduced in the two terms will be of opposite sign, and the quantity S', the *computed spectral slit width*, can be defined.

$$S' = \Sigma + \Pi \tag{6}$$

S' can be calculated precisely for any Littrow spectrometer operating under selected conditions. This quantity probably provides the best basis we now possess for comparing the resolution of different makes and types of prism spectrometers and adjusting them to operate under comparable conditions. Σ and Π can be calculated if the mechanical slit widths, focal length, and prism dimensions of the spectrometer are known; some manufacturers provide graphs from which these functions can be obtained very simply.

The effect of the finite slit width is to broaden the absorption band and to lower the intensity at the maximum, as is illustrated for the carbonyl band of camphor in FIGURE 1.

To take account of this effect, a series of "apparent" intensity units was introduced:[12]
the apparent molecular extinction coefficient

$$\epsilon_\nu^{(a)} = \frac{1}{cl} \log_{10} \left(\frac{T_o}{T} \right)_\nu \tag{7}$$

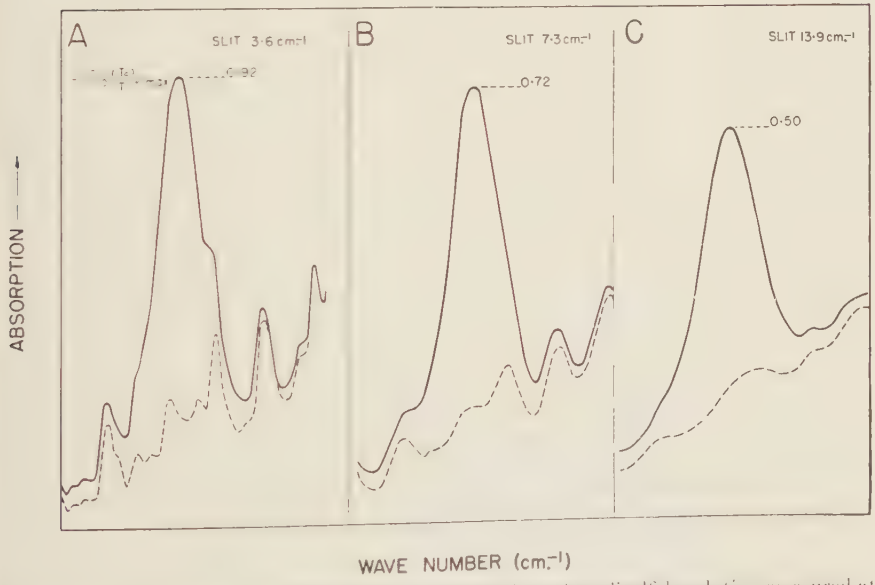


FIGURE 1. Carbonyl stretching band of camphor in carbon disulfide solution measured at different spectral slit widths on a single-beam spectrometer. The broken line is the I_o curve showing atmospheric water vapor bands. (Reproduced by permission of Interscience Publishers, Inc.[9])

the apparent integrated absorption intensity

$$B = 2.303 \int \epsilon_{\nu}^{(a)} \cdot d\nu \tag{8}$$

and the apparent half intensity band width ($\Delta\nu_{1/2}^{(a)}$), the width of the band where $\epsilon^a = 0.5\ \epsilon_{\text{max.}}^{(a)}$.

In EQUATION 7, $\log_{10}(T_o\ T)_\nu$ is defined as the absorbance observed when the spectrometer is set at the wave number ν, but the radiation is not assumed to be monochromatic. More recently these definitions have been broadened to take into account the other sources of error that affect the measured band intensity (Jones and Sandorfy,[9] pp. 276, 287).

It has been shown by Ramsay[12] that in many cases an isolated infrared absorption band in a liquid or solution, when measured under high resolution, can be fitted by a Lorentz curve

$$\log\left(\frac{I_o}{I}\right)_\nu = \frac{a}{(\nu - \nu_o)^2 + b^2} \tag{9}$$

where $a/b^2 = \log\left(\frac{I_o}{I}\right)_{\text{max.}}$, $2b = \Delta\nu_{1/2}$, and ν_o is the wave number of the band maximum.

On the basis of this assumption Ramsay was able to relate the spectral slit errors to the ratio $S'/\Delta\nu_{1/2}^{(a)}$. For $S'/\Delta\nu_{1/2}^{(a)} = 0.2$ these calculations indicate $\epsilon_{\text{max.}}/\epsilon_{\text{max.}}^{(a)} = 1.03$. For several reasons,[7] it is convenient to accept this 3 per cent error in $\epsilon_{\text{max.}}$ as the maximum that can be tolerated without the application of a specific finite slit correction. The absorption bands of organic compounds in solution are seldom narrower than 8 to 10 $cm.^{-1}$, and it follows that the spectral slit correction need not be applied if the spectrometer can be operated at computed spectral slit widths in the range 1.6 to 2.0 $cm.^{-1}$ or below.

The considerations that limit the validity of this criterion, and its significance in measurements made with the conventional types of small prism spectrometers used under routine laboratory conditions are considered in another publication.[7] The broad conclusions can be summarized as follows:

(1) Adequate resolution to eliminate the need for slit corrections for solution measurements on bands broader than 8 $cm.^{-1}$ can be obtained with prism spectrometers using a large double monochromator or a double-pass single-prism monochromator with a full set of LiF, CaF_2, and NaCl prisms, provided each prism is used most efficiently over its range of optimum dispersion. A grating spectrometer is preferable, since it will provide more energy at the same spectral slit width if the rest of the optics are comparable.

(2) If the more commonly employed single-prism (double-beam) spectrometers are used, the spectral slit width will vary widely over different regions of the spectrum; the manufacturers usually report the resolution at the wave number at which the spectral slit width is minimal. Provided these spectrometers are operated at high gain, with the full set of prisms, computed spectral slit widths in the range 2 to 4 $cm.^{-1}$ should be achieved. Under these conditions the spectral slit errors will be serious only for the narrower

bands, such as the out-of-plane C—H deformation bands of aromatic and unsaturated compounds.

(3) Appreciable errors in $\epsilon_{max.}$ will occur if the conventional commercial spectrometers are used with a sodium chloride prism at wave numbers above about 1400 cm.$^{-1}$ For example, a C=O stretching band at 1720 cm.$^{-1}$ with $\epsilon_{max.}^{(a)}$ of 420, as measured at a computed spectral slit width of 6.9 cm.$^{-1}$, will increase to 485 when Ramsay's correction is applied.

(4) Double-beam spectrometers, in which the energy in the control beam is maintained constant by a servomechanism that opens the slits when passing through a solvent os atmospheric absorption band, provide rapidly varying resolution in scanning the spectrum, and are not well adapted for infrared intensity work.

Other sources of error in intensity measurements. Errors caused by scattered radiation in the spectrometer are of considerable importance, as they can be quite large and are liable to vary considerably in different spectrometers of the same general design. They are, therefore, one of the major causes of variation in intensity measurements made on different spectrometers operated at comparable slit schedules. The presence of appreciable scattered radiation can be detected in passing through regions of very strong absorption, where it will cause the curve to flatten to a plateau below the 100 per cent absorption line. Corrections for this error can be applied,[13] but it is better to use a double-prism monochromator or a double-pass single-prism monochromator, in which the scattered radiation should be extremely small.

Another source of error is associated with electrical and mechanical inertia in the recording system. This can become serious if the spectrum is scanned too rapidly to allow the recorder pen to follow the proper curve, but can be reduced by adjusting the cell length and concentration so that all measurements of absorbance fall within the range 0.2 to 0.8. The limiting scanning speed should be determined experimentally by selecting the steepest absorption band and traversing it at progressively slower speeds until further speed reduction fails to alter the peak intensity. It is obvious that accurate measurements of cell thickness and concentration are essential; the spectra should always be run in duplicate to check on gross concentration errors, and a separate solvent control spectrum should be recorded on each curve.

If these precautions are followed, there should be no difficulty in obtaining reproducibility of $\epsilon_{max.}^{a}$ similar to that shown in TABLE 1 for the spectrum of deoxytigogenin (FIGURE 23). These measurements were made independently by two operators at an interval of two years using a Perkin-Elmer Model 112 double-pass single-beam spectrometer. The major source of variation is probably the uncertainty in the evaluation of the absorbance at the band maxima. This is caused by the high noise level (about 1.5 per cent), which cannot be avoided if slit schedules sufficiently narrow to eliminate appreciable spectral slit errors are to be maintained.

The Use of Integrated Absorption Intensities

The methods for computing integrated absorption intensities have been described in detail,[12, 14] including the extrapolation techniques to correct for

Table 1

Comparison of Duplicate Intensity Measurements on the Infrared Spectrum of Deoxytigogenin*

Operator M.A.M Nov. 23rd, 1954		Operator J.L.M. Nov. 29th, 1956	
$\nu_{max.}$	$\epsilon^{(a)}_{max.}$	$\nu_{max.}$	$\epsilon^{(a)}_{max.}$
1263 cm.$^{-1}$	30	1263 cm.$^{-1}$	36
1240	137	1238	138
1207	36	1205	40
1173	167	1173	164
1156	102	1156	100
1096	90	1096	92
1072	248	1070	240
1055	305	1055	300
1020	84	1021	85
1003	126	1003	125
992	87	991	94
981	332	980	323
970	100	969	100
961	121	960	120
951	49	950	50
922	155	922	162
899	237	898	239

* The spectra were measured in carbon disulfide solution by different operators on a Perkin-Elmer Model 112 double-pass single-beam spectrometer.

spectral slit errors when the measurements are made under low dispersion. The use of integrated absorption intensity measurements is limited to bands that are free of appreciable overlap effects. Most extensive studies have been made on the C=O stretching bands,[11-17] while other bands that have been investigated include the C–O stretching bands near 1200 cm.$^{-1}$ in esters,[16a, 16b, 18-19] the N–H stretching bands of amines,[20] the C≡N stretching bands of nitriles,[21, 22] and the various C–H stretching and deformation bands of hydrocarbons, ketones, and esters.[16a,b]

The integrated absorption intensity of the C=O stretching bands varies over a range of about 1 to 5 intensity units, although the range for other types of bands can be much greater; thus Thompson and Steel[22] record the intensity of C≡N stretching bands over a 180-fold range in different types of nitriles, and Russell and Thompson[20] record a 130-fold range of intensity for N–H stretching bands in amines.

For C=O stretching bands, the intensities tend to increase with factors that lower the C=O stretching frequency, such as conjugation, and to fall with factors that raise the C=O frequency, such as α-halogenation. However, the relation between C=O frequency and band intensity is not a simple one. Attempts to investigate it in substituted acetophenone derivatives were unsuccessful. Although the position of the C=O stretching maximum in these compounds can be related to the reactivity of the carbonyl group as measured by Hammett's σ function, the band intensities show no similar relationship.[23] Barrow[17] has shown that for some conjugated carbonyl groups the C=O stretching band intensity varies with the resonance energy of con-

jugation. For the aromatic nitriles, Thompson and Steel[22] have demonstrated that log A varies linearly with the Hammett σ constant for the aromatic substituent. However, for the present at least, these remain isolated observations. As far as the organic chemist and biochemist are concerned, the integrated absorption intensities should be treated for the present as empirical quantities, and their use in determination of molecular

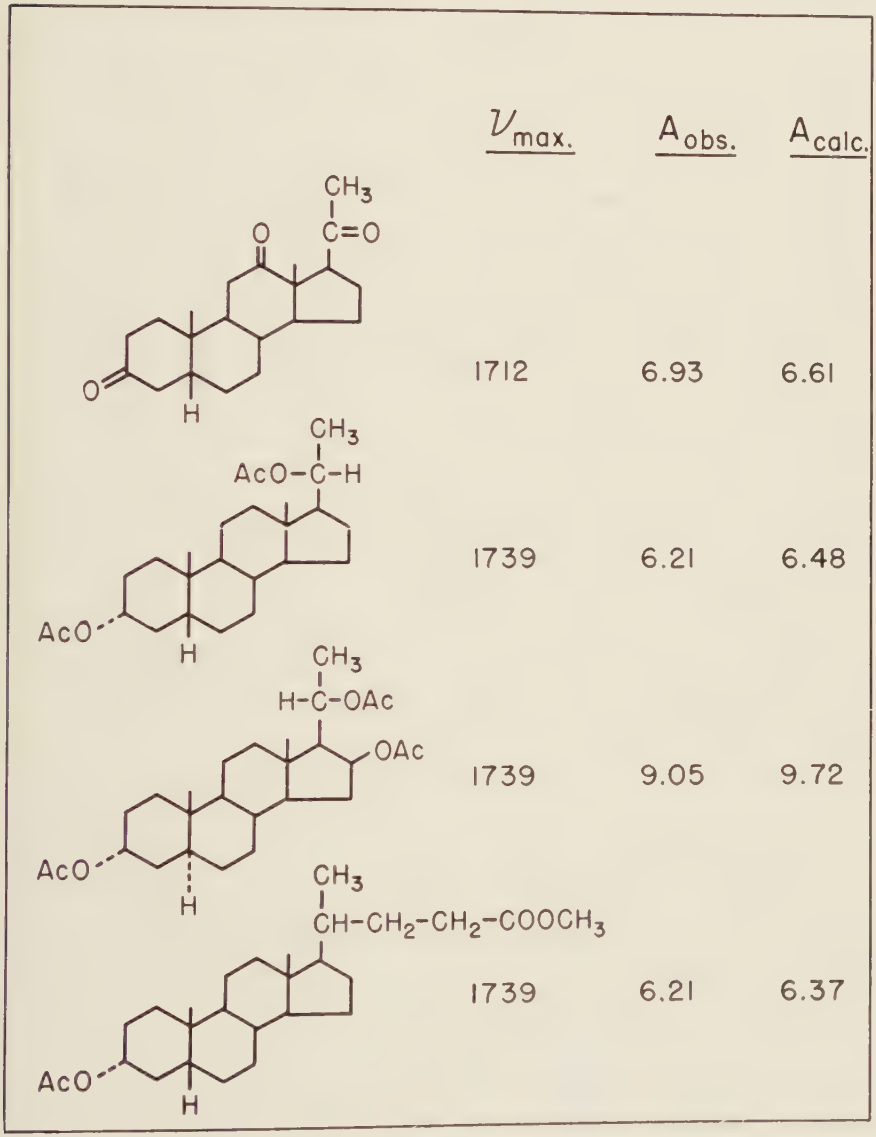


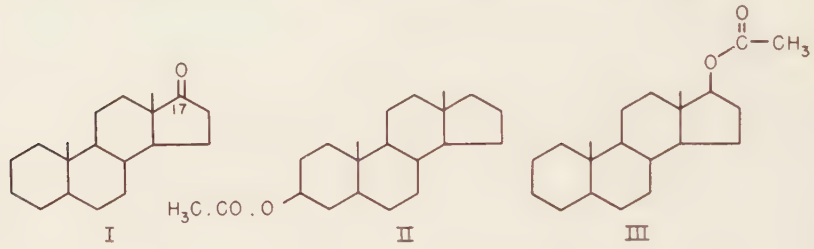
FIGURE 2

structure should be based on comparisons between compounds of known and unknown structure.

Studies with steroids have shown that the integrated absorption intensities of the C=O stretching bands remain fairly constant for a carbonyl group in a given molecular environment, and this can be used for structure identification; it is particularly useful where several carbonyl groups are present and the bands overlap so that the individual maxima cannot be resolved.

An example is provided by the spectra of the four steroids shown in FIGURE 2, which exhibit only one absorption peak assignable to C=O stretching vibrations; the intensity measurements obtained by summing the values for the individual bands in the monocarbonyl compounds, however, clearly distinguish the number of carbonyl groups present.

In some circumstances simultaneous measurement of intensities in different regions of the spectrum can help to differentiate among alternative molecular structures. The 17-ketosteroid group (I) and the 3- and 17-acetate groups (II, III) (FIGURE 3) all absorb near 1740 cm.$^{-1}$ in CS_2 solution, with intensities of about 2.7 units for the ketone and 3.25 for the acetate groups. Intensity measurements in the C=O stretching region would not be precise enough to distinguish between the keto-ester and diester structures IV and V (FIGURE

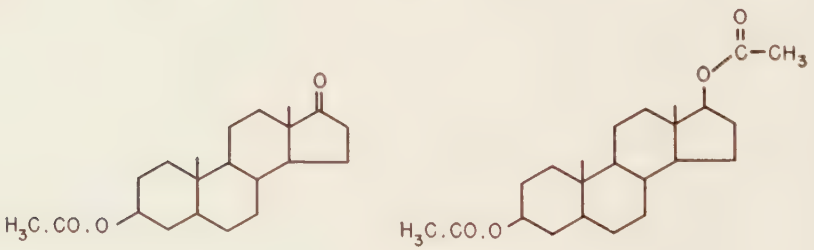


I A_{1740} 2.7; $A_{1190\text{-}1290}$ NIL

II A_{1740} 3.25; $A_{1190\text{-}1290}$ 4.0

III A_{1740} 3.25; $A_{1190\text{-}1290}$ 4.0

FIGURE 3



IV A_{1740} 5.95; $A_{1190\text{-}1290}$ 4.0

V A_{1170} 6.5; $A_{1190\text{-}1290}$ 8.0

FIGURE 4

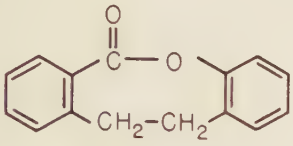
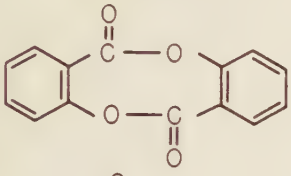
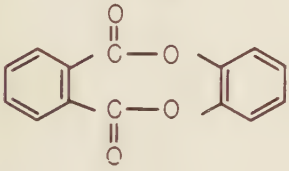
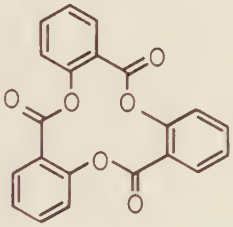
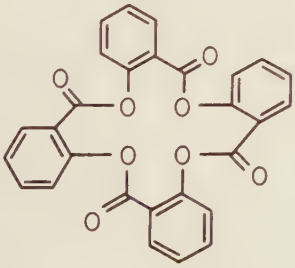
	No. of C=O GROUPS	INTENSITY per CARBONYL
	1	5.0
	2	4.9
	2	4.1
	3	4.0
	4	3.6

FIGURE 5

4), but the acetate also absorbs near 1240 cm.$^{-1}$, and integration over the range 1190 to 1290 cm.$^{-1}$ gives a value of 4 units per acetate group and negligibly small values for a ketone. The structures IV and V can therefore readily be distinguished by the dual set of intensity measurements, as indicated below the formulas. It may be noted also that, if the acetate group in II is axial, the 1240 cm.$^{-1}$ band splits into two or three components, but the integrated absorption intensity over the 1190 to 1290 cm.$^{-1}$ region embraces all the components and the summed intensity is not affected significantly on this account.[18]

Another illustration of the use of integrated absorption intensities for characterizing molecular structure is provided by some investigations on condensation products of catechol and phthalic acid carried out in collaboration with Wilson Baker and W. D. Ollis of Bristol University.[24] Baker, Ollis, and their collaborators, by the self-condensation of salicylic acid and the condensation of catechol with phthalic acid under varied conditions, have prepared the compounds shown in FIGURE 5. The structures of these compounds have been established by methods not involving infrared spectrometry; the measurements of the integrated absorption intensities over the whole carbonyl region give the values shown in the table, ranging from 3.6 to 5 units per carbonyl group. One of the products of the condensation of catechol and phthalic anhydride, ψ-catechol phthalate, has a molecular formula $C_{14}H_8O_4$, but the chemical evidence cannot distinguish the alternative structures VI and VII (FIGURE 6), of which VII is a conformational isomer of the third structure in FIGURE 5. The value of 4.9 for the total integrated absorption intensity obtained for the C=O band of ψ-catechol phthalate would be 4.9 per carbonyl on the basis of structure VI and 2.45 per carbonyl for VII, which strongly supports the monocarbonyl structure for this compound.

Under other conditions catechol and phthalic anhydride react to yield a product of molecular formula $C_{28}H_{18}O_8$, dicatechol phthalate, for which structures VIII and IX (FIGURE 7) may be considered; although the distinction is here not quite as clear-cut as between VI and VII, the carbonyl band intensity favors the dicarbonyl structure IX.

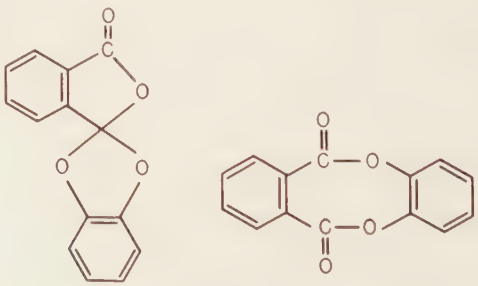


FIGURE 6

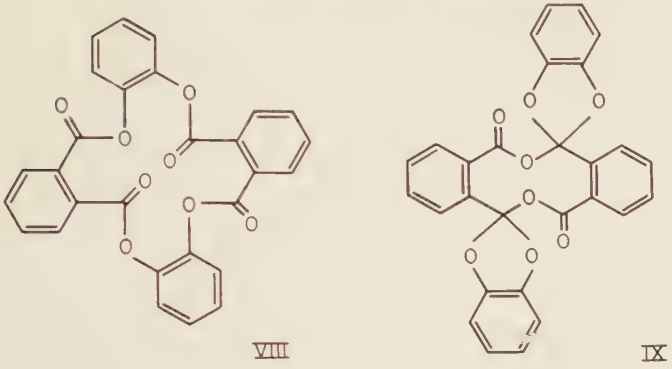


FIGURE 7

There is no doubt that increasing use will be made of integrated absorption intensities in molecular structure determinations as the factors determining the intensities become better understood. In the biochemical field it is rather fortunate that both the C=O stretching band and the N—H stretching bands are suitably located for such treatment.

Before leaving the subject of integrated absorption intensities, we should point out that such area measurements also have useful applications in purely analytical work and that infrared spectrometers with integrating attachments to record the apparent integrated absorption intensity over any preselected region of the spectrum are commercially available. The potential advantages of analytical techniques for multicomponent analyses based on integrated absorption intensity measurements are discussed by Rogoff in another paper in this publication.[25]

The Use of Molecular Extinction Coefficients

It has been noted above that the integrated absorption intensity can be related to the change of the electric moment during the vibration. Since the band area will be some product function of $\epsilon_{max.}$ and $\Delta\nu_{1/2}$, it follows that if $\epsilon_{max.}$ instead of A is taken as a measure of the band intensity, a tacit assumption is made that $\Delta\nu_{1/2}$ remains constant. This is not generally true, and the C=O stretching bands of steroids demonstrate clearly that the integrated absorption intensity measurements vary in a more regular fashion with molecular structure than do the maximal molecular extinction coefficients (Jones and Sandorfy,[9] p. 469; Jones *et al.*[14]).

In many parts of the spectrum, however, it is not possible to measure the areas under individual absorption bands, because of overlap with neighboring absorption; under these circumstances, $\epsilon_{max.}$ provides the best means available for evaluating band intensities. Provided these limitations are kept in mind, the $\epsilon_{max.}$ measurements can be made to reveal a good deal about the molecular structure and the nature of the molecular vibrations.

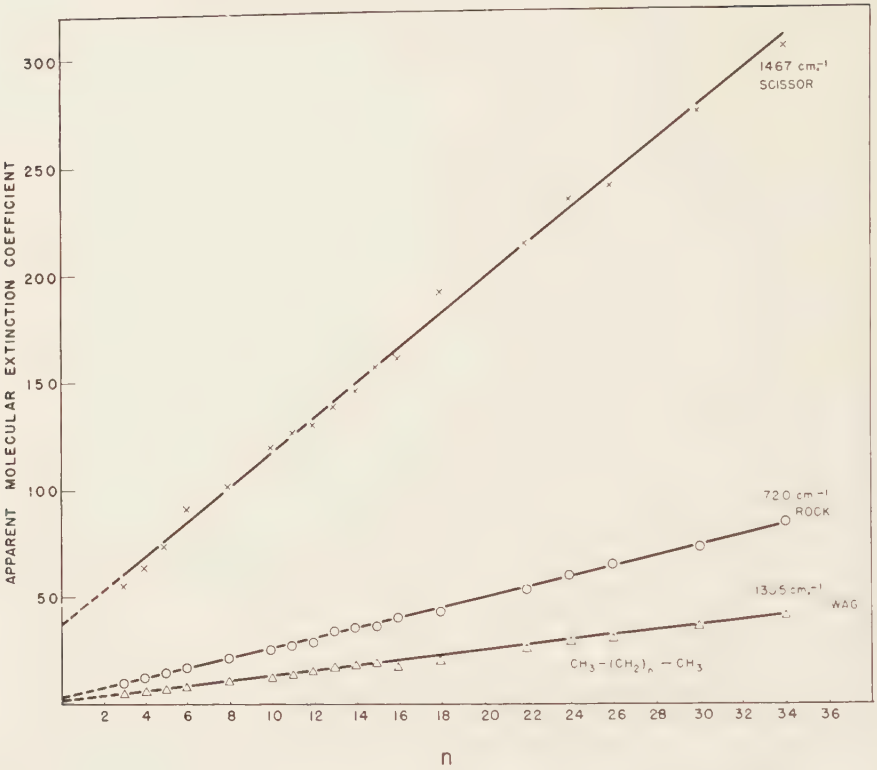


FIGURE 8. Effect of chain length (n) on $\epsilon^{(a)}_{max.}$ for methylene group vibrations of normal paraffin hydrocarbons. The 1467 cm.$^{-1}$ band was measured in CCl_4 and the other bands in CS_2 soln. (Reproduced by permission from *Record of Chemical Progress*.[28])

The homologous series of *n*-paraffin hydrocarbons is the simplest group of compounds in which regularities between $\epsilon_{max.}$ and molecular structure can be demonstrated. In FIGURE 8 the $\epsilon_{max.}$ values for the C—H deformation bands of the methylene groups of the *n*-paraffin hydrocarbons are plotted against the number of methylene groups in the chain. It is evident that each added methylene group makes an equal increment to the total intensity of these bands. The 1305 cm.$^{-1}$ and 720 cm.$^{-1}$ lines for the wagging and rocking vibrations extrapolate back to the origin, indicating that these vibrations are responsible for all significant absorption at these frequencies, but the 1467 cm.$^{-1}$ line for the scissoring vibration makes a large positive intercept with the zero ordinate axis, showing that other absorption also contributes to the band. In FIGURE 9 similar plots for the 1467 cm.$^{-1}$ methylene scissoring bands of homologous series of fatty acids are shown. This line resembles that for the *n*-paraffins and has approximately the same slope. This indicates that the addition of a polar group to the polymethylene chain has little effect on the vibration, which must therefore be highly localized within the methylene group.

Also shown in FIGURE 9 is the plot of $\epsilon^{(a)}_{max.}$ versus chain length for the

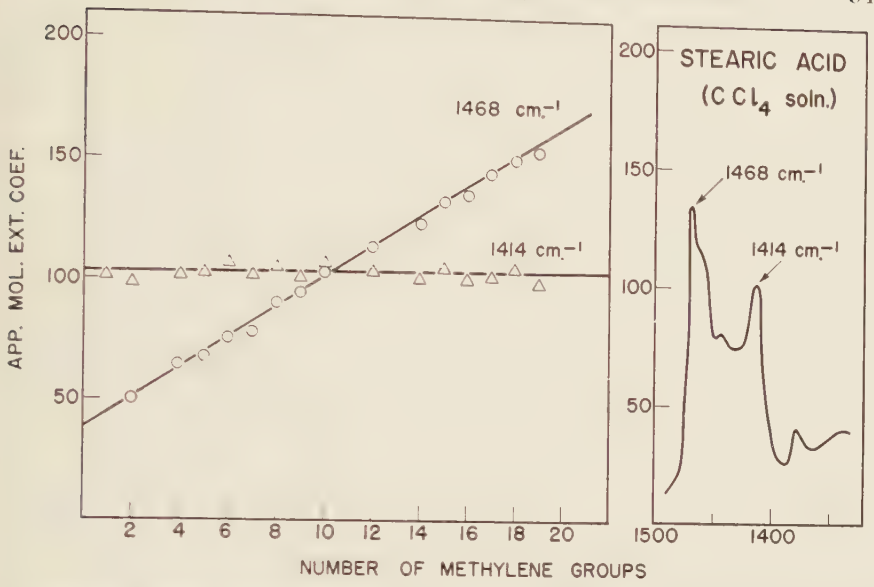
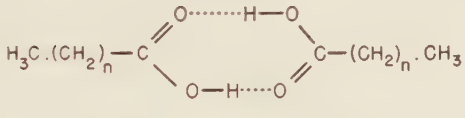


FIGURE 9. Effect of chain length on $\epsilon''_{max.}$ for absorption bands in straight-chain fatty acids. CCl_4 soln.



X

FIGURE 10

1414 cm. $^{-1}$ band of normal fatty acids. These compounds are almost completely dimerized in carbon disulfide solution (X) (FIGURE 10), and the insensitivity of the 1414 cm. $^{-1}$ band intensity to chain length indicates that this band must be due to a vibration localized in the carboxylic acid dimer group; this is in agreement with the assignment made by Hadži and Sheppard.[26]

The ϵ'', ν plots can be used effectively in differential and summational curve analysis to sort out absorption associated with different parts of a complex molecule. This can be done experimentally by using equimolar solutions in matched cells with a double-beam spectrometer, or by algebraic addition of appropriate ϵ'' ν curves. Both methods have their merits and, in the absence of intermolecular effects, give similar results.

A simple example of this technique is provided by the spectra of the deuterated ethyl acetates in the C H stretching region shown in FIGURE 11.[27] In the nondeuterated acetate all five of the bands indicated in FIGURE 11A are assigned to vibrations localized in the methylene and methyl radicals of the —O—CH_2– CH_3 group, and it is assumed also that there is negligible interaction between the methylene and methyl group vibrations. This is

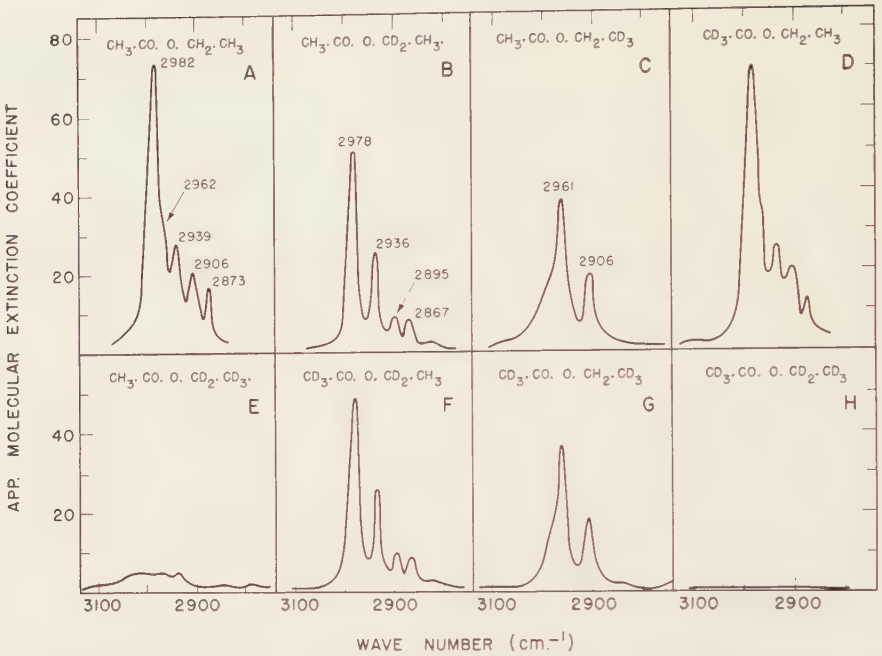


FIGURE 11. Infrared spectra of deuterated ethyl acetates in the C—H stretching region CCl_4 soln. (Reproduced by permission from *Canadian Journal of Chemistry*.[21])

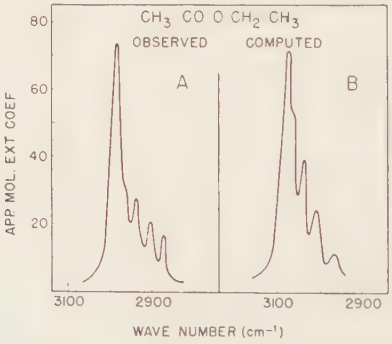


FIGURE 12. Comparison of C—H stretching region of ethyl acetate (curve A) with summation curve for $CH_3{\cdot}CO{\cdot}O{\cdot}CD_2{\cdot}CD_3 + CD_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CD_3 + CD_3{\cdot}CO{\cdot}O{\cdot}CD_2{\cdot}CH_3$ (curve B). CCl_4 soln.

confirmed by comparison of the spectrum of the light ester in FIGURE 12 with the curve obtained by adding the ϵ_{ν}'' values for the spectra of $CD_3{\cdot}CO{\cdot}O{\cdot}CD_2{\cdot}CH_3$, $CD_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CD_3$, and $CH_3{\cdot}CO{\cdot}O{\cdot}CD_2{\cdot}CD_3$ at 1 $cm.^{-1}$ intervals across the spectrum.

The absorption of the same compounds in the C—D stretching region is shown in FIGURE 13, but when the spectrum of the fully deuterated ester is compared with the summation spectrum for $CD_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_3 + CH_3{\cdot}$

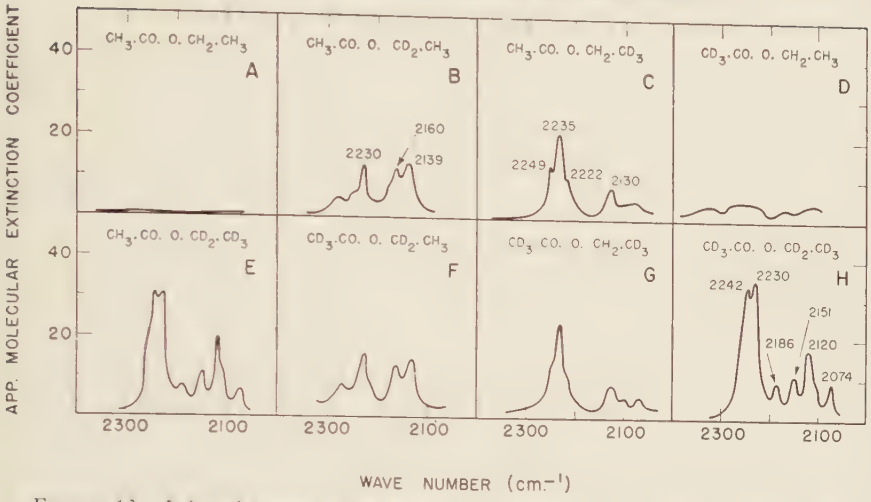


FIGURE 13. Infrared spectra of deuterated ethyl acetates in the C—D stretching region CCl4 soln. (Reproduced by permission from *Canadian Journal of Chemistry*.[27])

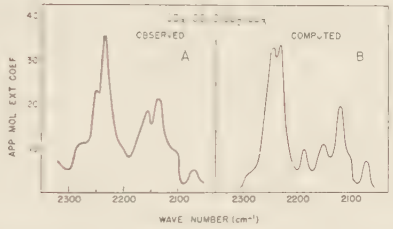


FIGURE 14. Comparison of C—D stretching region for fully deuterated ethyl acetate (curve A) with summation curve for $CD_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_3 + CH_3{\cdot}CO{\cdot}O{\cdot}CD_2CH_3 + CH_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CD_3$.

$CO{\cdot}O{\cdot}CD_2{\cdot}CH_3 + CH_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CD_3$, shown in FIGURE 14, significant differences are apparent, particularly between 2200 and 2100 cm.$^{-1}$ Here we are evidently not dealing with a simple summation of superimposed $>CD_2$ and $-CD_3$ vibrations, and probably there is interaction with overtones of strong skeletal vibrations near 1200 cm.$^{-1}$

Although incidental to the above argument, the plots of ϵ/ν in FIGURES 11 and 13 also demonstrate that the absorption associated with the methyl group in the acetyl radical is weaker by a factor of ten than that of the methyl group in the aliphatic environment of the ester ethyl.

Absorption between 1475 and 1350 cm.$^{-1}$ In more complex natural products the spectral region between 1475 cm.$^{-1}$ and 1350 cm.$^{-1}$ is particularly suited for this type of differential intensity analysis, as most of the absorption stems from C—H deformation vibrations of aliphatic systems, and these are largely localized vibrations. Aromatic ring systems do not normally absorb strongly in this region and, although deformation vibrations of hydroxyl groups occur, they are comparatively weak. Several group frequencies associated with nitrogen atoms may affect this region of the spectrum but in the main they

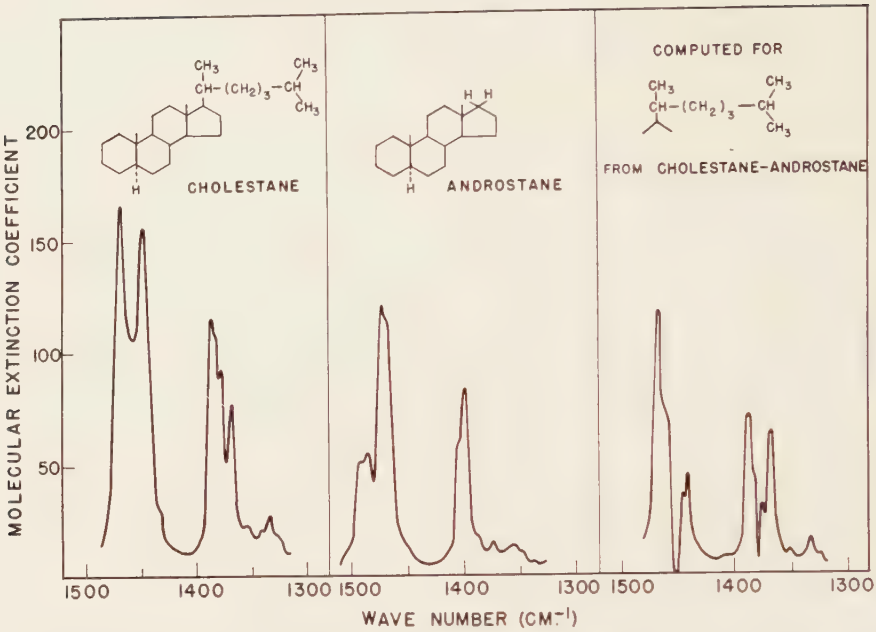
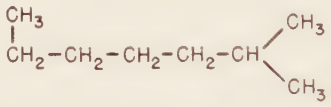


FIGURE 15. Differential spectrum for cholestane side chain. CCl_4 soln. (Reproduced by permission from *Record of Chemical Progress.*[28])



XI

FIGURE 16

involve types of groups not normally encountered in natural products (for example, nitrosamines, nitramines, nitrate ions, and alkyl azides).

The separation of the absorption associated with different parts of a complex molecule can be illustrated by the example of the cholestane side chain, which can be evaluated by subtracting the androstane spectrum from the cholestane spectrum, as shown in FIGURE 15. The curve for 2-methylheptane (XI) (FIGURE 16) is shown in FIGURE 17 for comparison. The doublet absorption between 1400 and 1350 cm. $^{-1}$ associated with the terminal isopropyl group of the side chain is clearly apparent in the cholestane-androstane differential spectrum with an intermediate band that can be assigned to the C_{21}-methyl group absorption. The strong minimum at 1450 cm. $^{-1}$ corresponds in position with the scissoring vibration of the methylene groups of cyclopentane and results from the loss of the C_{17}-methylene group on attachment of the side chain. Such differential curves can be used to predict the spectra of other cholestane derivatives if the spectrum of the corresponding androstane derivative is known, and examples have been published elsewhere (Jones and Sandorfy,[9] p. 364; Jones[28]).

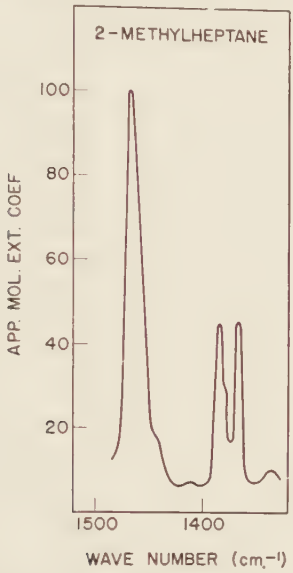


FIGURE 17. Infrared spectrum of 2-methylheptane. CCl_4 soln.

The effect of introducing a second substituent into the steroid ring system can often be determined in a similar fashion, provided the two substituents are well separated. The spectra of the disubstituted compounds androstan-3β-ol-17-one acetate and etiocholan-3α-ol-17-one acetate shown in FIGURE 18 are simulated closely by the algebraic addition curves for androstan-3β-ol acetate + androstan-17-one − androstane, and etiocholan-3α-ol acetate + etiocholan-17-one − etiocholane. Other examples of the same kind have also been published.[28]

Absorption below 1350 cm.$^{-1}$ At lower frequencies (1350 to 650 cm.$^{-1}$) the spectrum is complicated by the skeletal vibrations, and some appreciation of the intensities associated with the various types of groups is helpful in interpreting this part of the spectrum.

One of the most important applications of infrared spectrometry has been the differentiation of isomeric aliphatic hydrocarbons, such as *n*-hexane and 2-methylpentane, the spectra of which are shown in FIGURE 19. If the terminal methyl group of these hydrocarbons is replaced by a carboxylic acid group, most of this specificity is lost (FIGURE 20). The intensity of the absorption, however, is enhanced tenfold, and it is the strong absorption associated with the carboxylic acid dimer group that largely overwhelms the weaker but specific absorption associated with the aliphatic chain.

The spectra of saturated steroid hydrocarbons below 1350 cm.$^{-1}$ seldom contain bands with $\epsilon^{(a)}_{max.} > 25$, but on the introduction of oxygen-containing functional groups the intensity increases. Ketosteroids exhibit bands with $\epsilon^{(a)}_{max.}$ up to 100, steroid alcohols up to 250, and acetates up to 700.[28, 29]

It is not possible to formulate a detailed picture of the vibrations responsible for this strong and complex absorption, but it is evident that the charge dis-

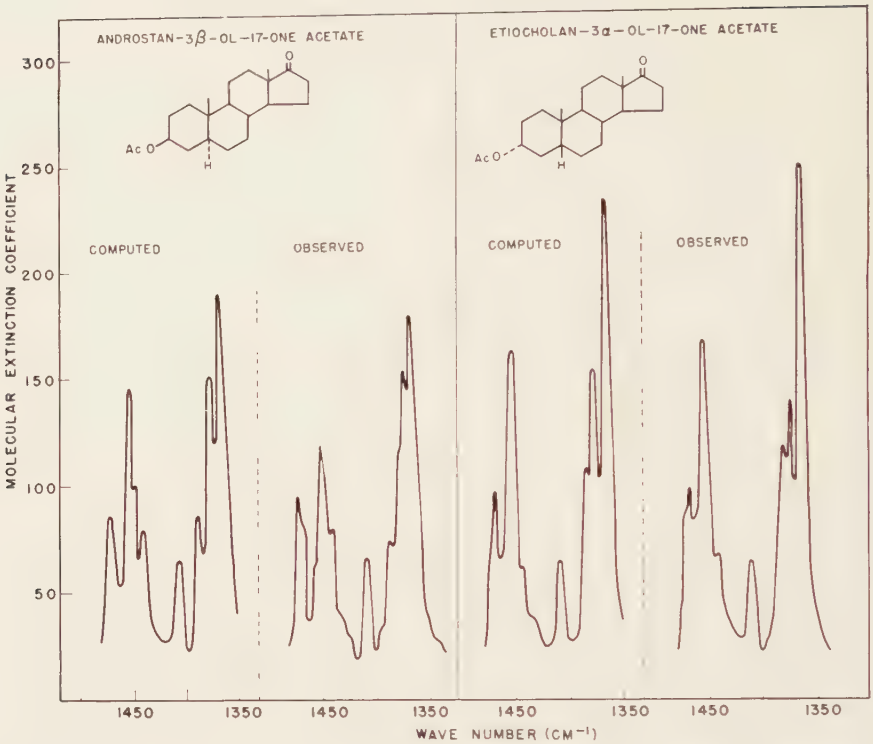


FIGURE 18. Comparison of observed and computed spectra for 3-acetoxy-17-ketosteroid isomers. CS_2 soln.

placements associated with asymmetrical motions of carbon-oxygen and hydrogen-oxygen bonds will be much greater than those associated with similar motions in purely alicyclic systems. Since, in this part of the spectrum, we are dealing principally with vibrations of the skeleton, we cannot treat these strongly infrared-active vibrations as highly localized in individual bonds or small molecular groups, as they will be coupled into more general skeletal vibrations; the neighboring carbon atoms will also carry enhanced charges through inductive effects. It is convenient to regard this strong absorption as originating in a *characteristic vibrational zone* of the molecule; this will confer a definite pattern of absorption on the spectrum, but it will not have such high spectral specificity as we associate with absorption more definitely localized in a small molecular group or individual linkage.

If two characteristic vibrational zones are present in the same molecule their effect on the spectrum will be approximately additive, provided they are sufficiently remote from each other to prevent strong coupling of their vibrations; this coupling may occur either through the bonds or directly by dipole-dipole interaction across space. The characteristic molecular zone vibrations will define the general appearance of the spectrum, but the weaker vibrations in other parts of the molecule will induce sufficient secondary perturbations

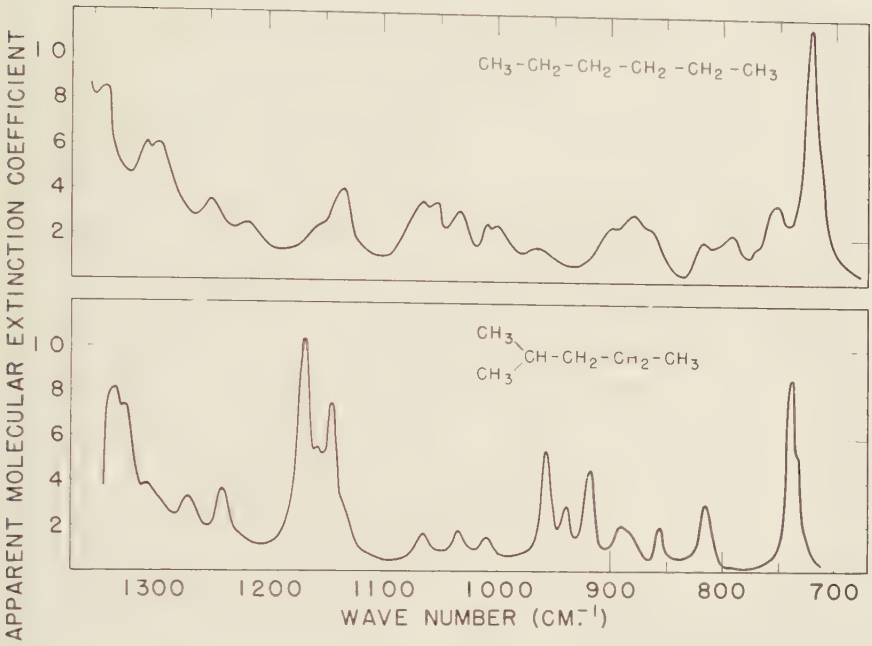


FIGURE 19. Effect of chain branching on the "fingerprint" spectra of aliphatic hydro carbons. CS_2 soln. (Reproduced by permission of Interscience Publishers, Inc.[9])

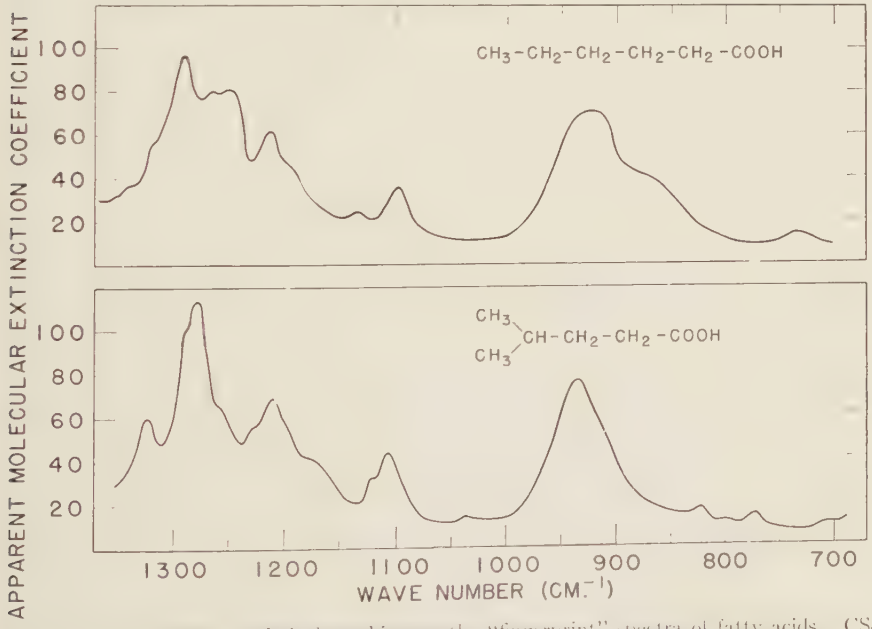


FIGURE 20. Effect of chain branching on the "fingerprint" spectra of fatty acids. CS_2 soln. (Reproduced by permission of Interscience Publishers, Inc.[9])

to maintain the "fingerprint" specificity associated with absorption in this region of the spectrum.

The effects of small structural changes on spectra dominated by characteristic vibrational zones can often be predicted by differential curve analysis similar to that employed in the 1475 to 1350 cm.$^{-1}$ region. Good examples of the domination of spectra by two characteristic vibrational zones are provided by the 3-hydroxy-17-ketosteroids. The 3α- and 3β-hydroxy groups in the 5α- and 5β-steroid series constitute four distinctive vibrational zone absorp-

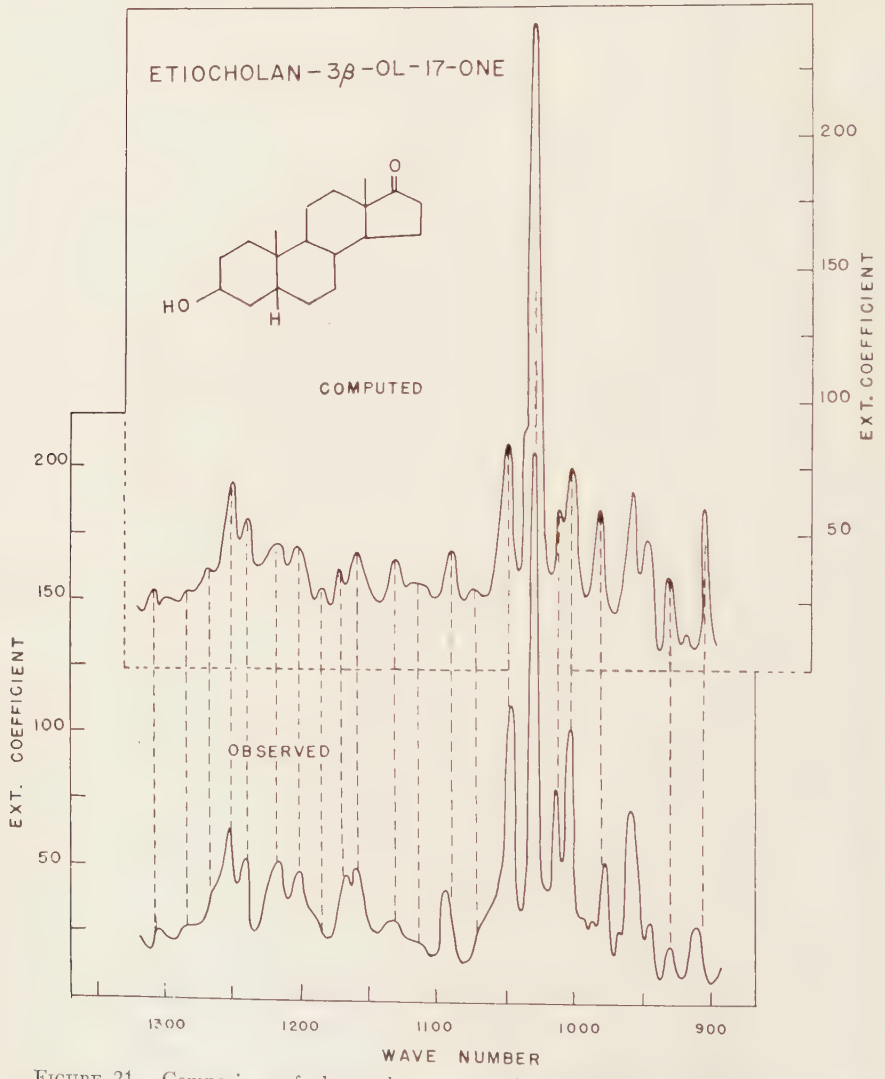
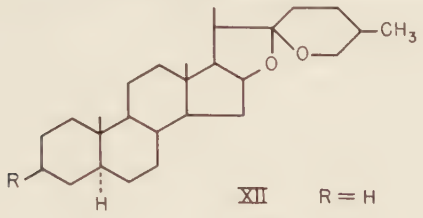


FIGURE 21. Comparison of observed spectrum of etiocholan–3β–ol–17–one with curve computed from etiocholan 3β–ol + etiocholan–17 one – etiocholane. CS_2 soln.



XII R = H

XIII R = OH

FIGURE 22

tions which combine with the 17-ketone absorption to define the major features of the spectra of these compounds. The simulation of "fingerprint" spectra by these techniques was first demonstrated[29] for androstan-3α-ol-17-one by algebraic summation of the spectra of androstan-3α-ol + androstan-17-one − androstane. The spectrum of another of these isomers, etiocholan-3β-ol-17-one, computed from etiocholan-3β-ol + etiocholan-17-one − etiocholane, is shown in FIGURE 21. Other examples have also been published,[28,30] and the problem is now being investigated systematically.

The side chain group of cholestane behaves as a weak but characteristic vibrational zone, while that of the bile acid methyl esters is much stronger. Most striking, however, is the spiroketal system of the steroidal sapogenins (FIGURE 22), which is associated with extremely strong absorption. The steroidal sapogenins derived from natural saponins contain at least one functional group in the steroid ring system. The absorption associated with these substituents modifies the absorption between 1075 and 975 cm.$^{-1}$, but the remainder of the spectrum below 1350 cm.$^{-1}$ is determined principally by the spiroketal zone. When these spectra were initially examined[31] 3-deoxytigogenin (XII), the parent compound of the isosapogenin series, had not been prepared, but it was possible to predict the spectrum from that of tigogenin (XIII) by the algebraic summation of tigogenin − androstan-3β-ol + androstane. Subsequently, through the kindness of C. R. Eddy, a sample of 3-deoxytigogenin was obtained, and the observed and predicted spectra of this compound are compared in FIGURE 23.

The use of characteristic vibrational zones is necessarily based on measurements of molecular extinction coefficients. Up to the present its validity has not been investigated with compounds other than steroids, which are particularly well suited for this kind of analysis. It would seem probable that similar behavior would be found in other types of molecules built upon rigid and predominantly alicyclic skeletons, such as triterpenes and alkaloids. However, it is unlikely to apply to aromatic systems where the substituent can modify the bond character at considerable distances because of the much greater electron mobility. It may also be much less effective in predominantly noncyclic aliphatic molecules, or in monocyclic systems, in which there is greater freedom for free rotation about single bonds. In such compounds the substituent will have a much greater steric effect in determining the equilibrium conformation of the molecule, and the spectrum will be considerably affected by such conformational changes in the carbon skeleton.

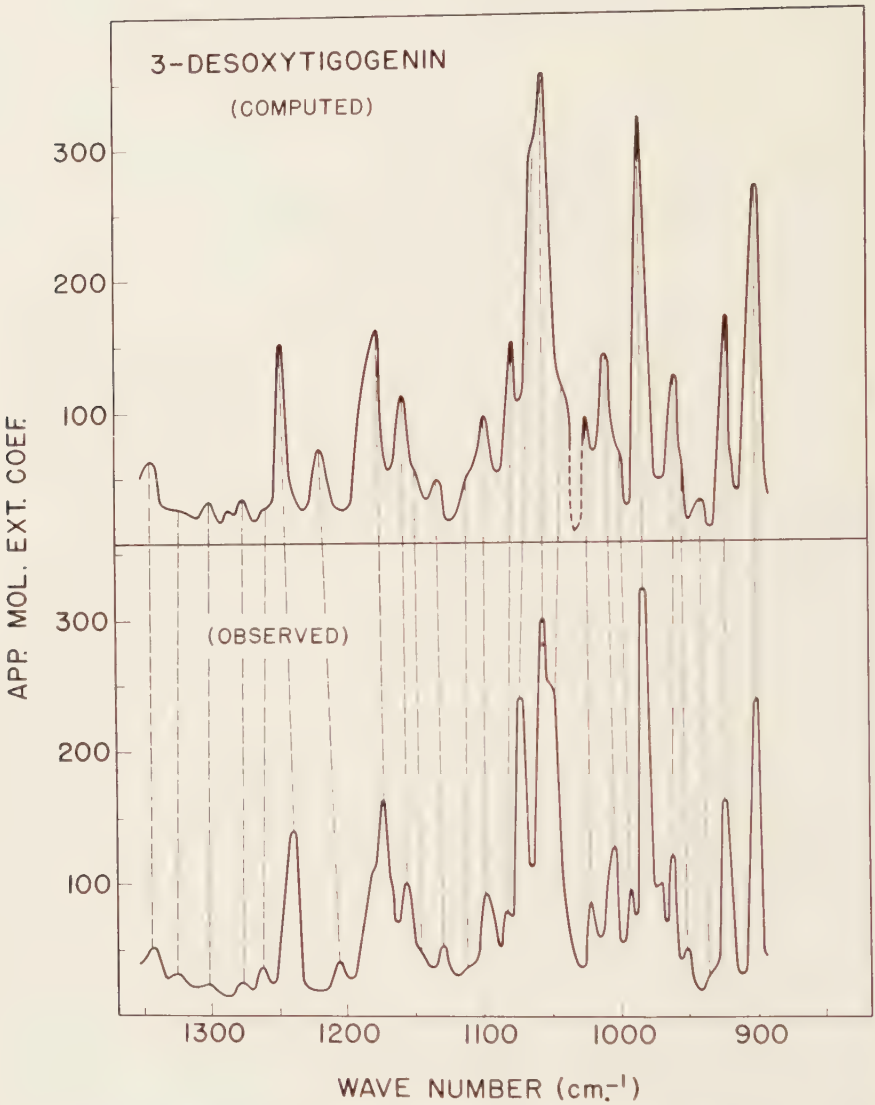


FIGURE 23. Comparison of observed spectrum of 3-deoxytigogenin with predicted spectrum. CS_2 soln.

Conclusions

The principal object of this article has been to demonstrate that both molecular extinction coefficients and integrated absorption intensities serve in different ways to extend the information about molecular structure that can be derived from purely qualitative studies of infrared absorption spectra.

It is clearly not feasible to put all infrared spectrometry on an absolute intensity basis. The necessity of working with dilute solutions limits such

studies to compounds that are soluble in the limited range of infrared-transmitting solvents and, using the equipment at present available, such measurements are slow and tedious. Nevertheless, it must be recognized that the infrared spectrum has now become the most important single physical constant for the characterization of an organic compound, and we badly need libraries of these spectra recorded in a form suitable for rapid and accurate transfer of data between laboratories and for coding and sorting by electronic means.[25] Plots of ϵ against ν would have many advantages for this purpose, and it should not be unduly difficult to introduce the $1 \cdot \mathrm{cl} \cdot \log_{10}(x)$ function into the amplifier and record the ϵ ν plot directly. The larger prism spectrometers now commercially available are just about capable of achieving sufficient resolution to justify such measurements, if used at their maximum efficiency. Grating spectrometers that could easily meet the limiting resolution requirements could be produced commercially if sufficient demand were once established.

Pending these developments, all who are concerned with recording the infrared spectra of pure compounds for documentation should at least be fully acquainted with the limitations of their experimental technique and with the various factors that influence the accuracy of infrared intensity measurements. Unless this is done, it may happen that many of the spectrograms now being incorporated into catalogues of spectra will fail to meet the more exacting specifications that future applications may demand.

Acknowledgment

We are very grateful to A. S. Hay, R. Lauzon, Mrs. M. A. MacKenzie, J. L. Mateos, and A. Nadeau, whose assistance with the measurement of spectra played an important part in the experimental work on which this paper is based.

We also wish to express our thanks to T. F. Gallagher and his colleagues at the Sloan-Kettering Institute for Cancer Research, New York, N. Y., with whom we have enjoyed the opportunity of frequent discussion of these problems.

References

1. Rose, F. W., Jr. 1938. J. Research Natl. Bur. Standards. **20:** 129.
2. Anderson, J. A., Jr. & W. D. Seyfried. 1948. Anal. Chem. **20:** 998.
3. Hampton, R. R. & J. E. Newell. 1949. Anal. Chem. **21:** 914.
4. McMurry, H. L. & V. Thornton. 1952. Anal. Chem. **24:** 318.
5. Cross, L. H. & A. C. Rolfe. 1951. Trans. Faraday Soc. **47:** 354.
6. Reggiani, M., B. Casu & G. Caroti. 1955. Gazz. chim. ital. **85:** 1058.
7. Jones, R. N. 1957. Spectrochim. Acta. In press.
8. Bunsen, R. W. & G. Kirchhoff. 1861. Pogg. Ann. **113:** 337.
9. Jones, R. N. & C. Sandorfy. 1956. The application of infrared and Raman spectrometry to the elucidation of molecular structure. *In* Technique of Organic Chemistry. A. Weissberger, Ed. **9:** 247. Interscience. New York, N. Y.
10. Thompson, H. W., 1955. Molecular Spectroscopy. : 94. Institute of Petroleum. London, England.
11. Williams, V. Z. 1948. Rev. Sci. Instr. **19:** 135.
12. Ramsay, D. A. 1952. J. Am. Chem. Soc. **74:** 72.
13. Opler, A. 1950. J. Opt. Soc. Am. **40:** 401.
14. Jones, R. N., D. A. Ramsay, D. S. Keir & K. Dobriner. 1952. J. Am. Chem. Soc. **74:** 80.
15. Richards, R. E. & W. R. Burton. 1949. Trans. Faraday Soc. **45:** 874.

16a. Francis, S. A. 1950. J. Chem. Phys. **18:** 861.
16b. Francis, S. A. 1951. J. Chem. Phys. **19:** 942.
17. Barrow, G. M. 1953. J. Chem. Phys. **21:** 2008.
18. Jones, R. N., P. Humphries, F. Herling & K. Dobriner. 1951. J. Am. Chem. Soc. **73:** 3215.
19. Russell, R. A. & H. W. Thompson. 1955. J. Chem. Soc. : 479.
20. Russell, R. A. & H. W. Thompson. 1955. J. Chem. Soc. : 483.
21. Skinner, M. W. & H. W. Thompson. 1955. J. Chem. Soc. : 487.
22. Thompson, H. W. & G. Steel. 1956. Trans. Faraday Soc. **52:** 1451.
23. Jones, R. N., W. Forbes & W. Mueller. 1957. Can. J. Chem. In press.
24. Baker, W., W. D. Ollis & R. N. Jones. Unpublished data.
25. Rogoff, M. 1957. Ann. N. Y. Acad. Sci. **69** (1): 27.
26. Hadži, D. & N. Sheppard. 1953. Proc. Roy. Soc. London. **A216:** 247.
27. Nolin, B. & R. N. Jones. 1956. Can. J. Chem. **34:** 1392.
28. Jones, R. N. 1955. Record Chem. Progr. Kresge-Hooker Sci. Lib. **16:** 271.
29. Cole, A. R. H., R. N. Jones & K. Dobriner. 1952. J. Am. Chem. Soc. **74:** 5571.
30. Jones, R. N., B. Nolin & G. Roberts. 1955. J. Am. Chem. Soc. **77:** 6331.
31. Jones, R. N., E. Katzenellenbogen & K. Dobriner. 1953. J. Am. Chem. Soc. **75:** 158.

THE INFLUENCE OF MOLECULAR ENVIRONMENT ON INFRARED SPECTRA

By Ralph S. Halford
Department of Chemistry, Columbia University, New York, N. Y.

Problems of structure and arrangement of biochemicals, especially *in situ*, are fundamental in biology. Spectroscopy at all wave lengths provides an arsenal of weapons with which we may hope to attack these problems at the molecular level. Among these weapons those of infrared spectroscopy are notable for their power and versatility.

Infrared spectra originate with the mechanical vibrations of molecules or aggregates of molecules. These vibrations are often localizable to good approximation, mainly within certain functional groups such as N—H, O—H, or C=O, to mention but a few. In such cases the localization is sufficiently complete for the corresponding functional groups to be recognized by their associated spectral contributions, even when these groups are present in a wide variety of molecules or when these molecules exist in differing molecular environments. However, the spectral contribution associated with a functional group is more or less subtly altered by the nature of the rest of the molecule to which the group is attached or by the solvents or other environments in which the same molecule might be examined. Starting from some idealized state of completely localized oscillation, we can regard the functional group as a kind of probe with which we can study its environment. This environment can be considered for the moment as being partly intramolecular and partly extramolecular, although this distinction will become quite vague as we proceed.

Dependence of a vibration of a functional group on its intramolecular environment is the foundation of the powerful art of qualitative chemical analysis through infrared spectra. For example, the spectroscopic distinction between a carboxylic acid and an ester, familiar to all infrared analysts, illustrates the differing interactions of a carbonyl function with two different intramolecular environments. This concept is too widely known to require further development here.

A functional group ordinarily interacts more vigorously, through its attaching chemical bonds, with its intramolecular than with its extramolecular environment; consequently, this intramolecular influence comes more immediately to our attention. The well-recognized manifestation of this predominant interaction usually is a small but easily detected shift in the spectral frequency associated with the functional group.

In general, the frequencies of molecular vibrations, which determine the frequencies of the radiation that they absorb, are almost negligibly affected by extramolecular environment. For this reason we must look elsewhere among the characteristics of the spectrum for the manifestations of extramolecular environment. By the same token, we can add to the store of available probes of molecular structure furnished by functional groups all of

the other, unlocalizable vibrations of molecules. These latter vibrations involve the whole molecular skeleton and furnish the spectral "fingerprints" whereby two different molecules containing the same functional groups usually can be differentiated.

Spectral contributions from all sources, skeletal as well as functional-group vibrations, provide probes equally useful for the study of extramolecular environment. Extramolecular environment exerts its influence primarily upon the intensity and upon the contour or fine structure of the absorption envelope associated with each molecular vibration. An illustration of the variety of these manifestations and some hints concerning their significances can be offered first in terms of the simple substance benzene.

In the region of 800 to 1200 cm.$^{-1}$, benzene vapor exhibits only one rather intense band of absorption, centered near 1037 cm.$^{-1}$, with P and R branches arranged symmetrically on either side. The mere addition of a nonabsorbing gas, such as N_2, will cause the band to appear progressively shallower and broader until finally, at only rather moderate pressures of N_2, the P and R branches are no longer clearly discernible.

If the original amount of benzene vapor is liquefied,[1] the fine structure corresponding to the P and R branches disappears completely, although the width of the band and the location of its center otherwise remain about the same. More important, *six* new absorptions of lesser but nevertheless quite appreciable intensity emerge at about 850, 975, 990, 1010, 1145, and 1175 cm.$^{-1}$

When the liquid benzene is frozen, the first, third, and last of these new absorptions disappear again; the persistent ones at 975, 1010, and 1145 appear to become intensified, while all three of them, along with the principal absorption near 1035, become spectacularly sharpened.[2,3] This sharpening of the envelopes is so pronounced that the residual widths in each case are properties primarily of the ordinary monochromator rather than of the substance, and it is perhaps better practice to designate the absorptions in solid benzene as lines. Finally, in the solid the absorption near 975 cm.$^{-1}$ acquires a fine structure consisting of a pair of clearly resolved lines.

These observations with benzene, and their known counterparts with a number of other simple substances, demonstrate emphatically that the extramolecular environment can exert profound and varied influences upon an infrared spectrum. When the foregoing observations with benzene are subjected to an appropriate theoretical analysis, it is possible to conclude with a high degree of confidence that the environment in solid benzene is well ordered, centrosymmetric, and anisotropic about the center of each and every benzene molecule, while the environment in liquid benzene is disordered and devoid of symmetry locally, but statistically isotropic on the average taken over a larger sample of molecules.

Let us proceed now to another example. The simple molecule 1,2-dichloroethane has the interesting feature that it can exist for brief periods of time in either of two isomeric conformations: the two chlorine atoms can be either *trans* or *gauche* with respect to one another. The lifetimes of these conformations are sufficiently great so that each has its own characteristic infrared

spectrum. The spectrum observed with either the vapor or liquid of this substance, wherein both isomeric conformations coexist in comparable proportions, is a superposition of the separate spectra for the two kinds. When this substance is frozen, however, the resulting crystal consists exclusively of molecules of the *trans* kind. The spectrum for the crystal becomes so markedly different from the ones for liquid or vapor that an experienced infrared analyst might insist (quite rightly), when comparing them without other knowledge, that the spectra were from two different substances. This striking spectroscopic difference originates nevertheless in a mere partial rotation about a carbon-carbon single bond, a process whose counterpart occurs many times over whenever a polypeptide skeleton coils up randomly, folds, extends fully, or winds into a helix in the course of changing interactions with different environments. Speaking loosely, one might liken the mixture of conformations in liquid 1,2-dichloroethane to a random coil, while the all-*trans* crystal might be likened to a fully extended polypeptide chain. The spectra for proteins in different conformations surely must be different to a recognizable degree.

There is more to be learned from the simple substance 1,2-dichloroethane. At extremely low temperatures, the molecules form a well-ordered crystal wherein the environment is centrosymmetric, but anisotropic about the center of each molecule. As the temperature is elevated, the crystal becomes disordered by a process whereby a whole molecule rotates rigidly through some angle about an axis passing through its two chlorine atoms, remaining all the while exclusively *trans*. This disordering process reaches its climax in a "lambda-point" at about $-95°$ C., about 40° below the melting point. Infrared spectra recorded[4] at various temperatures reveal that sharp lines observed at low temperatures become broadened as the disorder develops. At temperatures above the "lambda-point" the more prominently affected lines have broadened into bands having widths similar to corresponding ones in the liquid.

A valid and potentially useful generalization, supported by a variety of observations and having a firm although incomplete theoretical foundation, is that the extraordinary sharpness observed in crystal spectra originates with the long-range order characteristic of the perfect example of that state. As the range of order in an imperfect arrangement becomes short, even though only in some partial aspect, the spectrum ceases to be sharp, and absorption lines broaden into bands. In crystalline 1,2-dichloroethane the disorder is relatively mild and many aspects of long-range order persist, but the disorder nevertheless reveals itself immediately in the spectrum.

However, the spectra of crystalline 1,2-dichloroethane in the disordered state remain strictly in accord with our expectations for a centrosymmetric local environment, all evidences of disorder notwithstanding. We may thus conclude that this crystal consists of domains that are well ordered internally and are large enough to contain most of the molecules in interior positions, but these are disordered with respect to one another. Such is the potential power of our probes.

One more example will be included here to underline the sometimes peculiar

sensitivity of the probes that we are considering. Acetylene is a simple substance indeed, and its vapor exhibits an infrared spectrum that is both simple and well understood. Infrared spectra observed[5] with single crystals of acetylene are in certain respects perhaps the most complicated of any as yet encountered with crystals. Bands are broader even than those in the vapor spectrum; absorption envelopes are highly unsymmetrical; and there are indications of a wealth of poorly resolved fine structure in which we have been unable to discover any systematic pattern. The crystal structures (two forms) are simple and well ordered, insofar as they are known. Acetylene consists, however, of *ortho-* and *para-* species, analogous to those encountered with hydrogen, in proportions of three to one. With these proportions, every molecule of acetylene in the crystal will have on the average at least one neighbor of the opposite species and, as a result, for certain subtle reasons the crystal is totally disordered dynamically. Moreover, the two species may even have distinct spectra, although the measure of their differences in the crystal remains to be determined. This is truly a very subtle peculiarity of environment, yet its existence is signaled with exceptional loudness by the infrared spectra.

It is clear from these examples that infrared spectra are quite significantly altered by changes of extramolecular environment. Further evidence for this can be found in the several reports that have appeared recently concerning the complications attending analyses and identifications of materials when polymorphic transitions are induced by grinding during the process of preparing mulls or KBr pellets for spectroscopic examination. Anyone who possesses a double-beam spectrometer can design and perform simple experiments in differential spectroscopy to convince himself that a mixture of two supposedly noninteracting solvents possesses a spectrum that is distinctly more than the superposition of the ones for its two pure components.[6,7]

What has all of this to do with biology? Proteins, nucleic acids, polysaccharides, and the like are gigantic molecules, and it is only prudent to ask now whether investigations of environmental effects in spectra, conducted with simple molecules such as 1,2-dichloroethane, benzene, or acetylene, have any bearing upon studies of these biochemicals.

Let us consider for the moment a simple polypeptide, such as polyglycine or polyalanine, of indefinitely large degree of polymerization, in a fully extended configuration. The molecule has a structure that repeats itself periodically along the chain. In short, the molecule is a "one-dimensional" crystal whose repeating unit contains just two identical residues. By analogy with three-dimensional crystals, where the theory of their spectra rests upon substantial foundations and periodicity of structure plays a prominent role, the spectrum of our polypeptide will be fully interpretable in terms of the structure of its repeating unit alone. Better yet, if we describe the repeating unit as a "unit cell" containing two identical residues, the analogy tells us that the spectrum will be fully interpretable in terms of the structure of only one residue, with added refinements (doubling of absorptions) that depend upon the arrangement of the two identical residues within the repeating unit. The rest of the chain constitutes, so to speak, an "extramolecular," crystalline environment

for one residue contained in it. This analogy has been developed systematically by Tobin[9] in a rigorous form suitable for application to all sorts of simple polymers, and has been studied in other ways by Primas and Günthard.[10, 11]

The backbone of our polypeptide contains strongly absorbing functional groups, whereas the appended remainder of the amino acid residue contains, in most cases, only weakly absorbing ones. Thus, the absorption localizable mainly within the backbone usually will predominate; so much so for many amino acids that the main features of the spectra for their fully extended polypeptides will appear to be identical. With this in mind, suppose now that we proceed to the consideration of a protein made up from a number of different amino acid residues. The minor features of a polypeptide spectrum, the ones contributed by its weakly absorbing side chains, will be multiplied in kind, but at the same time each will be diluted perhaps to the point of invisibility. The major features of the spectrum originate with the same backbone in either case, polypeptide or protein. In short, in the first and most important approximation there will be a common spectrum for all proteins whose backbones have similar conformations.

If amino acid residues of different kinds are arranged in a nonrepeating sequence in a protein, the protein in fully extended conformation can be likened to a partially disordered one-dimensional crystal. Bands in the common protein spectrum will be broader perhaps than those in a polypeptide; a doublet structure expected for absorptions in the polypeptide spectrum, originating with the presence of two identical residues in the repeating unit, may be masked or washed out by the broadening introduced into the spectrum of a protein by its side-chain disorder.

Up to this point we have been considering only a fully extended conformation of the backbone. If the backbone winds into a helix, the "common" protein spectrum will be altered in this new conformation, but the new spectrum will continue to be common to all proteins having the new conformation.

In summary, reasoning from results of studies conducted with crystals composed of simple molecules, we can expect rather confidently that the spectra of proteins will be quite simple in nature, consisting of a small number of broad bands whose locations and details of appearance are characteristic of the backbone conformation. The spectra as ordinarily examined will afford little if any information concerning either the specific identity of the protein or the identities and sequences of the amino acid residues contained in it. These conclusions appear to be well confirmed by experience.

A comparable example of this lack of spectroscopic differentiation, one having similar theoretical origins, was introduced in the previous paper by R. N. Jones, who remarked upon the striking similarities in the spectra of two acids as compared with the marked differences apparent in the spectra of their parent hydrocarbons.

It is time now to introduce some brief mention of infrared dichroism, that is, absorption anisotropy. This topic has been withheld deliberately from prior discussion because the concept of the individual probe is not so simply applicable to the phenomenon, and its improper use can lead us badly astray.

Here again, the investigation of small molecules is invaluable in pointing the way.

It is important to note first that absorption anisotropy in crystals composed of small molecules results always in the appearance of maxima and minima of absorption intensity measured along various crystallographic axes, regardless of how the axes of constituent molecules or their functional groups may be arranged with respect to the crystallographic frame. The phenomenon of absorption anisotropy is associated with the structure and motion of the entire crystal and cannot be localized within one of its parts. By the same token, the anisotropy observable in protein fibers, even if they are imagined to consist of perfectly arranged parallel helices or fully extended chains, is associated at the very minimum with the structure and mechanical behavior of the whole helix or chain and cannot be localized within one of its functional groups. The constituents of a crystal or of a large protein molecule oscillate in concert rather than individually, and the directional properties of the concerted oscillations are properties of the whole. The absorption intensity associated with each axis of a crystal or fiber belongs to a separate pattern of concert; consequently, the interactions of constituents with one another are different in each case. This circumstance results in a component of anisotropy over and above the one that we might attempt to assign to a localized constituent.

Directional properties of absorption measured with plane-polarized radiation belong to electrical oscillations that can be imagined to be generated locally in the first instance by mechanical oscillations. Dipoles appearing synchronously in proximity during concerted oscillations will induce added dipoles, each in the other charge distribution, whose directions always will be rotated toward the line joining centers of molecules. In this way the characteristic directions for electrical oscillations can become quite different locally from those for mechanical ones. This mechanism was proposed in our laboratory by Isao Ichishima to account for the results of observations conducted with crystalline naphthalene.

Both the molecular structure and the crystallographic arrangement of naphthalene are known from X-ray diffraction beyond any reasonable doubt. On grounds of symmetry, the directions permissible for mechanical oscillations within the isolated molecule are predicted confidently to be only three. The patterns for concerted oscillations in the crystal likewise can be predicted confidently from the known symmetry of the crystallographic arrangement. If all other considerations are ignored — corresponding in effect to a practice that has been employed in discussing infrared dichroism of proteins and that focuses attention upon local directions only — three cases of dichroic ratio can be predicted quantitatively for naphthalene.

Careful measurements show that the rich spectrum of naphthalene divides into three classes of dichroic behaviors, as expected, but in all three classes the quantitative behaviors deviate grossly from those predicted above. Perhaps the most meaningful measure of these deviations is contained in the statement that it would be necessary to rotate molecular axes by as much as 30° from their known orientations in the crystallographic frame in order to

achieve agreement between observations and predictions made in the usual manner. Clearly the naïve interpretation of absorption anisotropy would lead to a highly distorted picture of this crystal structure.

When the contributions from mutually induced dipoles are taken into account in the manner proposed by Ichishima, the effect of their inclusion is found to be equivalent to a rotation of the directions for electrical oscillations in the amount of about 30° away from the axes of the naphthalene molecule, and there is a satisfactory agreement between the newly predicted and the observed anisotropies of absorptions.

Since the lines joining centers of next-nearest functional groups of the same kind are neither parallel with nor perpendicular to the fiber axis in either a fully extended or a helical conformation, it is easy to see qualitatively that the mutual induction accompanying synchronous oscillations could operate to reduce the dichroism expected naïvely with either model of a protein. A quantitative statement can be made only after a decision concerning what is being electrically polarized and how polarizable it may be. Other problems enter into this.

Under the circumstances it is doubtful whether any quantitative significance can be attached at this time to attempted interpretations of observed absorption anisotropies in terms of protein structures. More effort is needed first in the area of small molecules.

Hydrogen bonding, an important and unique form of interaction with an environment, will be considered by another contributor to this publication.[12]

References

1. Halford, R. S. & O. A. Schaeffer. 1946. J. Chem. Phys. **14:** 141.
2. Mair, R. D. & D. F. Hornig. 1949. J. Chem. Phys. **17:** 1236.
3. Zwerdling, S. & R. S. Halford. 1955. J. Chem. Phys. **23:** 2221.
4. Gershenzon, M. 1957. Ph.D. Dissertation. Columbia Univ. New York, N. Y.
5. Krikorian, E. 1957. Ph.D. Dissertation. Columbia Univ. New York, N. Y.
6. Ketelaar, J. A. A. & F. N. Hooge. 1955. J. Chem. Phys. **23:** 749.
7. Fahrenfort, J. & J. A. A. Ketelaar. 1954. J. Chem. Phys. **22:** 1631.
8. Gellert, M. 1956. Ph.D. Dissertation. Columbia Univ. New York, N. Y.
9. Tobin, M. C. 1955. J. Chem. Phys. **23:** 891.
10. Primas, H. & Hs. H. Günthard. 1953. Helv. chim. Acta. **36:** 1659, 1791.
11. Primas, H. & Hs. H. Günthard. 1955. Helv. chim. Acta. **38:** 1254.
12. Liddel, U. 1957. Ann. N. Y. Acad. Sci. **69**(1): 70.

SOME SIMPLE HYDROGEN-BONDING SYSTEMS STUDIED BY INFRARED ABSORPTION

By Urner Liddel

Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md.

For some time biochemists and biologists have used the concept of "hydrogen bonding" in discussing molecular structure and molecular reactions. I believe the term hydrogen bond was first used by W. M. Latimer and W. H. Rodebush about 1920 to explain the peculiar properties of liquid water. N. V. Sidgwick also noted the unusual properties of certain hydrogen groupings and called this property "chelation" when, for example, the hydroxyl group in a compound did not have normal properties. Salicylaldehyde was a good example of this. Spectroscopic evidence for chelation was given in 1935[1] and for intermolecular action between alcohols in 1933.[2, 3]

Although much work has been reported since, and several reviews have been written,[4] it seems that no quantitative study of the simplest inter- and intramolecular bonding compounds has been published. It was felt that a good understanding of simple hydrogen-bonding systems was essential before an attack could be made on problems of real biological significance. Hence this study.

We have used a Perkin-Elmer Model 13 spectrometer with a LiF prism, modified to use linear frequency cams.[5] The accuracy of our measurements is about 3 $cm.^{-1}$ In the absorption coefficients reported, logarithms to the base 10 have been used.

The usual chemical purification procedures, such as distillation and recrystallization, were used. Purification of the alcohols was relatively straightforward. They were distilled over metallic sodium. In purification of the chelating compounds, considerable difficulty was encountered so that, although our physical measurements are quite accurate, the chemical composition of the samples remains in doubt. Aldol (β-hydroxybutyraldehyde) was purified by vacuum distillation, the sample being taken between 55° and 60° C. at a pressure of 12 to 17 mm. Hg. The freshly distilled material was immediately put in CCl_4 solution and examined. However, in the "pure" sample kept at 2° C. crystals formed in a few days. These crystals were separated, washed with CCl_4, dissolved, and the spectrum recorded. The resultant spectrum was nearly identical with that of the "pure" material. The literature states that aldol polymerizes, but one would not expect much polymerization to occur in the time elapsing between distillation and the first recording of the spectrum.

The dihydroxyanthraquinones were recrystallized from 95 per cent alcohol, and solutions were made from these crystals. The 1,2- and 1,4-substituted compounds exhibited unusual behavior on solution, even though the samples melted sharply at temperatures listed in the literature. Sufficient quinizarin (1,4-) was placed in a flask to form a 0.002 molar solution. The material did not all go into solution, even when the mixture was heated to 50° C. As a measure of desperation, considerably more material was added, the solution

filtered, and the spectrum recorded. When an aliquot of the remainder was evaporated to dryness, it was found that this solution was 0.0046 molar! Therefore, we must admit that we do not know that we are dealing with a "pure" material in any anthraquinone.

The temperature variation of the absorption was determined in a special cell constructed of block copper and controlled by water (or glycol solution) from an appropriate reservoir. The temperature of the cell was measured by a thermocouple imbedded in the copper surrounding the solution. The windows of the cell were fluorite and the space between the inner and outer windows was dried with magnesium perchlorate. The copper block extended on both sides of the absorption cell a distance greater than the diameter of the windows.

One of the greatest difficulties in this work was that of maintaining dry CCl_4. Various methods, including distillation through a 10-plate bubble-cap column, failed to dry it thoroughly. The method finally adopted consisted simply of placing beakers of the liquid in desiccators over P_2O_5 and evacuating. The resultant liquid showed about 2 per cent absorption at 3710 cm.$^{-1}$ in a 2 cm. cell. However, if this liquid was poured from one beaker to another in normal atmosphere the absorption increased to 10 or 20 per cent. Consequently, the solvent was removed from the desiccator with hypodermic syringes, and all the solutions were transferred in the same way. Even so, water was gradually absorbed by the solutions, so that they could not be kept for extended periods of time.

The spectrum of a 0.1 molar methanol solution in CCl_4 as a function of temperature is illustrated in FIGURE 1. It should be emphasized here that corrections have been made for the change in density of the solution as a function of

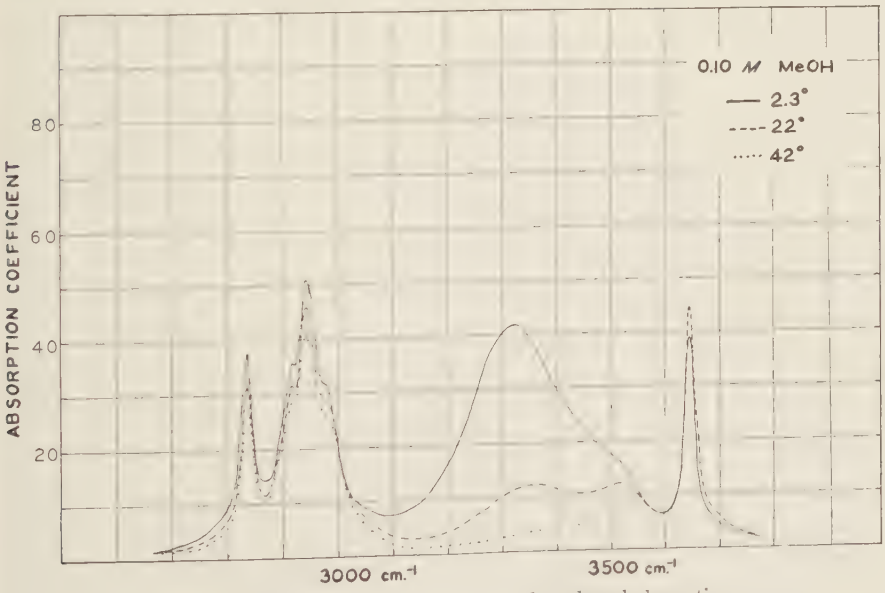


FIGURE 1. Temperature dependence of methanol absorption.

temperature so that these curves represent the true molar absorption coefficient. These curves illustrate not only the familiar changes in the monomer band at 3640 and polymer bands at 3515 and 3350 cm.$^{-1}$ but also the change in the CH bands around 2950 cm.$^{-1}$

We have adopted the usual assignment for the 3515 and 3350 cm.$^{-1}$ bands, namely, dimer and polymer, although the evidence does not appear to be conclusive. It seems odd that if a dimer configuration, be it cyclic or chain, gives a band at 3515, addition of one other molecule can reduce the frequency so much more. Moreover, there seems to be no peak formation that would indicate the presence of trimers, tetramers, etc. We find no evidence for these structures such as that found by Van Thiel, Becker, and Pimentel[6] at low temperatures.

It is obvious, however, from the spectra that the "polymer" absorption has practically disappeared at 42° C., and that it increases by nearly an order of magnitude as the temperature is lowered by 20°.

FIGURE 2 shows the spectra of solutions of methanol at 25° C. as a function of concentration. Here it would appear that the absorption in the "polymer" region is nearly linear with concentration. However, a careful plot of the data shows that it is not linear with concentration, or with the square or the square root of the concentration. The best fit was obtained when log a was plotted against log concentration, but even this fitted well over only a narrow range.

FIGURES 3 and 4 give the temperature and concentration dependence for ethanol solutions. The same general remarks apply as for methanol, except that here we did not see as great a change in the CH region. Only the band around 2920 cm.$^{-1}$ showed sensitivity to concentration and temperature. These data have been analyzed exhaustively to attempt a determination of the

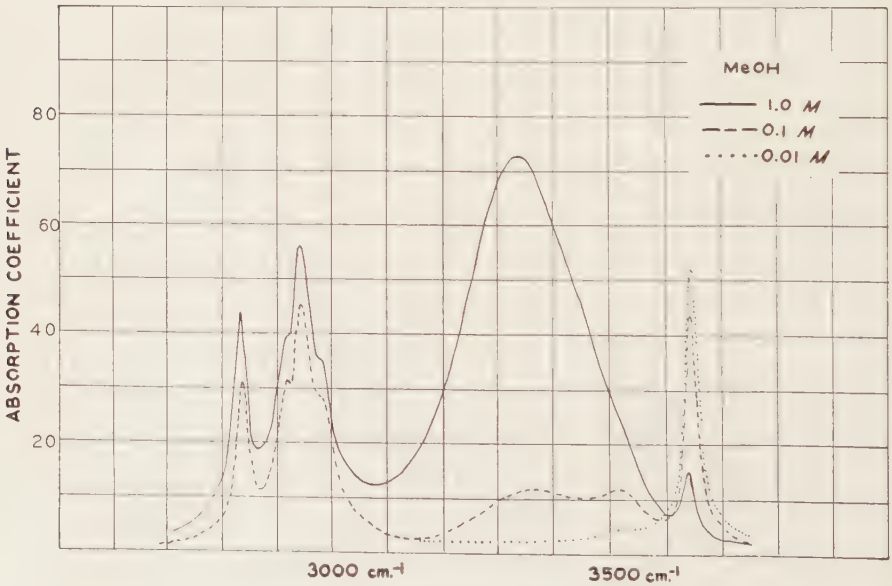


FIGURE 2. Concentration dependence of methanol absorption.

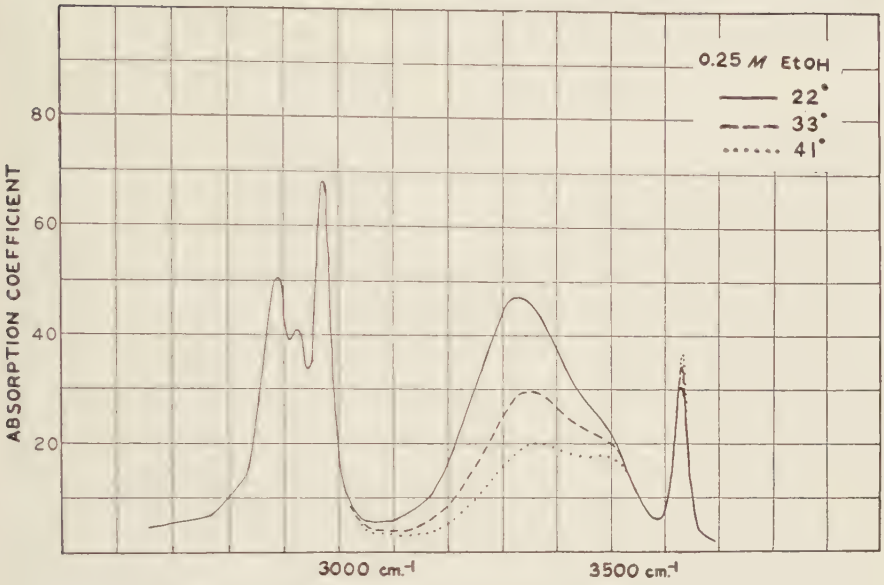


FIGURE 3. Temperature dependence of ethanol absorption.

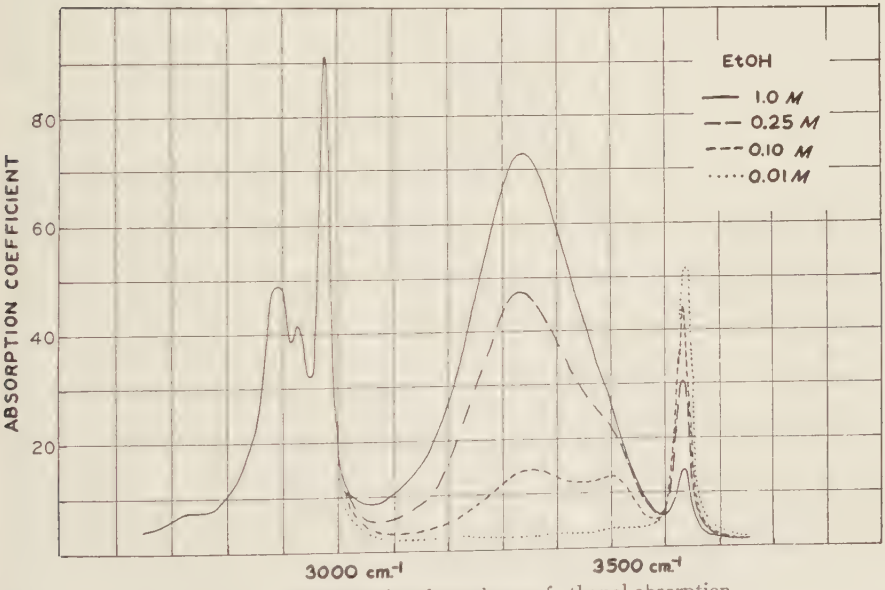


FIGURE 4. Concentration dependence of ethanol absorption.

heat of dissociation of the ethanol dimer. This will be the subject of a later paper. Suffice it here to say that the best value appears to be around 6 kcal./mole.

We also examined tertiary butanol as an example of a molecule that might

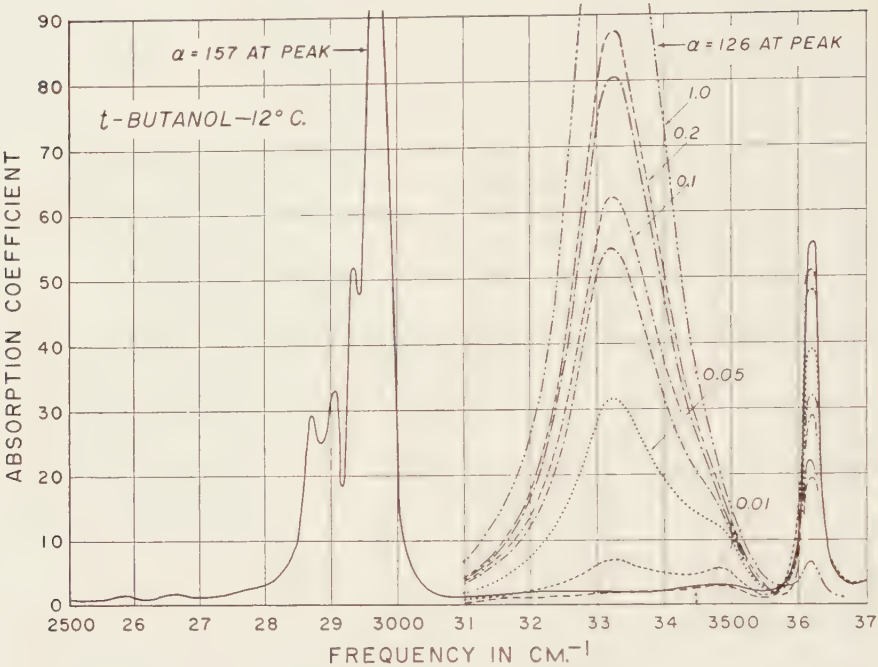


FIGURE 5. Concentration dependence of *t*-butanol absorption at −12° C. Concentrations, reading down, are 1.00, 0.204, 0.163, 0.100, 0.080, 0.051, 0.02, 0.01, and 0.005 molar.

be expected, because of its large hydrocarbon group, to interfere with the association. During the course of the work we discovered Coggeshall's study[7] of this molecule. He does not state the concentration range or temperature used. However, assuming that his data were recorded at 25° C., we obtained the same equilibrium constant for the monomer-dimer equilibrium. In FIGURES 5, 6, and 7 are shown our spectra for concentration dependence at −12° C., 25° C., and 50° C., respectively. In this instance, we found essentially no change in the CH region of the spectrum with alteration of temperature. Whenever large amounts of polymer were formed, a broadening of the band at 2905 cm.$^{-1}$ occurred. It was also noted that weak bands at 2590 and 2670 cm.$^{-1}$ showed a marked dependence on polymer formation, that is, the intensity of these bands increased with increasing polymer. There is no obvious explanation for this. In FIGURE 7 the absorption at 3500 cm.$^{-1}$ is greater than in any other spectrum and is of such a nature that it could not be "hidden" under the polymer band. This implies that the "dimer" structure is more stable than the polymer at high temperatures.

A major difficulty in computing such quantities as heats of dissociation lies in the fact that when no polymer band is apparent the intensity of the monomer band is also a function of temperature. The peak of the monomer absorption for methanol in CCl_4 seems to change 0.1 cm.$^{-1}$ per degree centigrade. Part of the change in intensity is due to change in the half width of the band, but this does not account for all of the effect. The observed changes are illustrated in

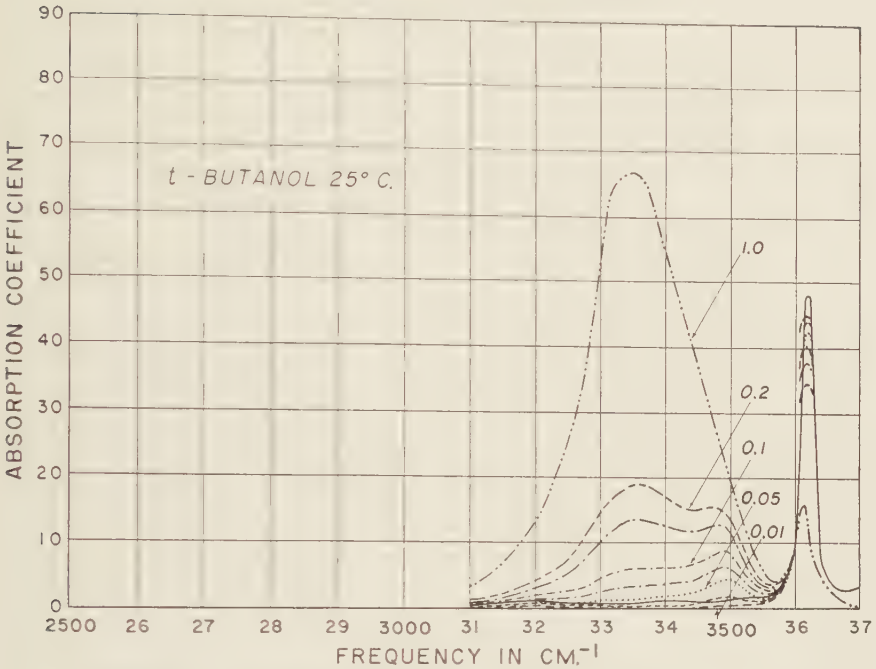


FIGURE 6. Concentration dependence of *t*-butanol absorption at 25° C.

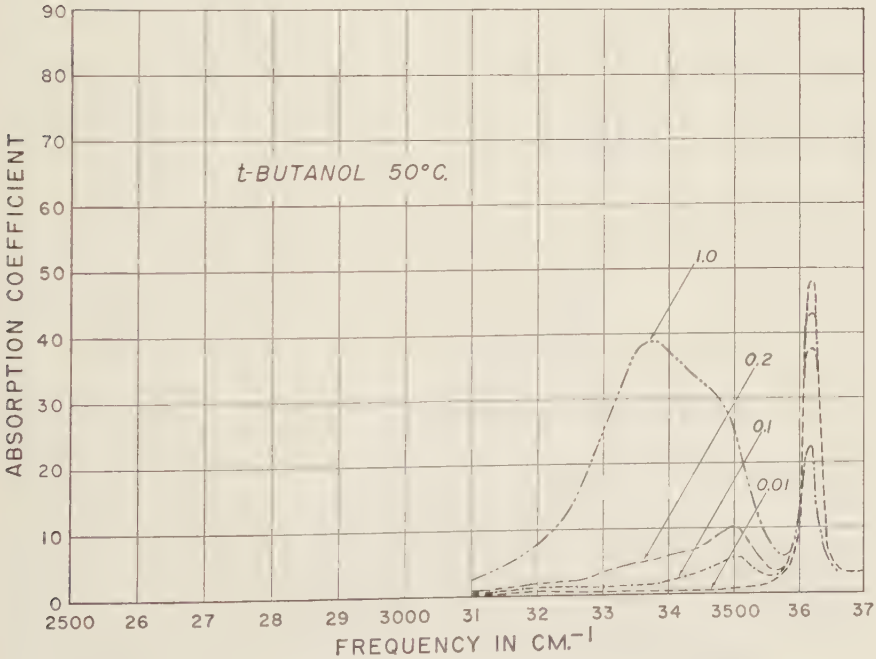


FIGURE 7. Concentration dependence of *t*-butanol absorption at 50° C.

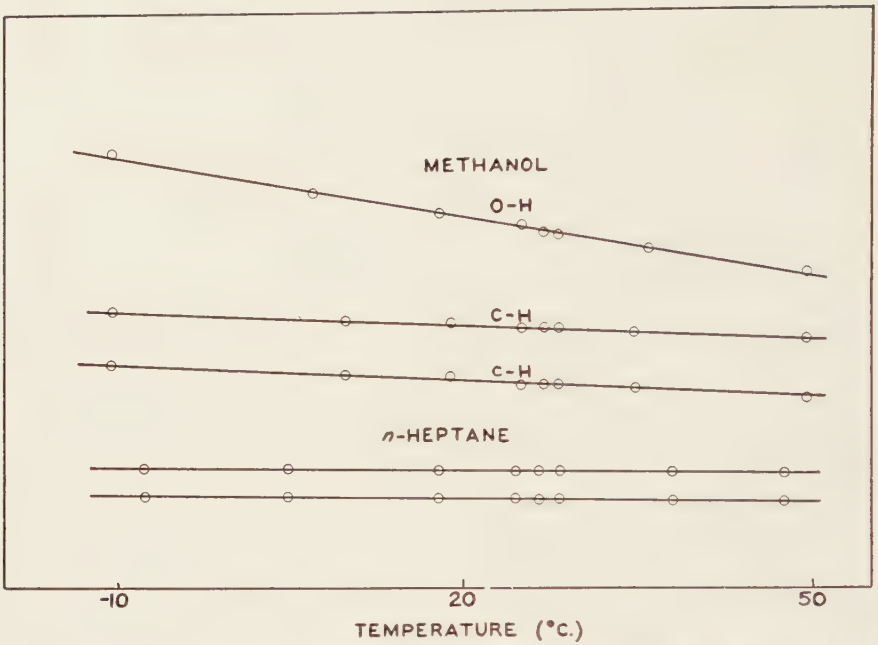


FIGURE 8. Temperature dependence of 3640, 2950, and 2835 cm.$^{-1}$ bands in methanol solutions and 2928 and 2960 cm.$^{-1}$ bands in *n*-heptane solutions.

TABLE 1
VARIATION IN INTENSITY OF ABSORPTION

Solute	Solvent	Conc. (*M*)	Frequency (cm.$^{-1}$)	R × 10^4 (deg.$^{-1}$)
CH_3OH	CS_2	0.005	3629	60
	CCl_4	0.005	3642–8	60
			2950	17
			2835	28
C_7H_{16}	CCl_4	0.005	2928	6
			2960	9
$CHCl_3$	CCl_4	0.05	3019	30
$CHCl_3$	—	12.6	3019	24

R is a negative slope of intensity in units versus T (°C.).

FIGURE 8 and tabulated in TABLE 1. The only explanation for this that occurred to us was that there was hydrogen-bond interaction of the OH and CH with the C—Cl of carbon tetrachloride. If the CH did interact, there should be a slight effect on the intensity of the bands of a normal hydrocarbon. We used *n*-heptane as an example and did find the postulated effect.

Huggins, Pimentel, and Shoolery[8a] concluded that measurements of proton magnetic resonance indicated a specific interaction involving the proton of chloroform, enabling it to form a hydrogen bond with other molecules. Huggins and Pimentel[8b] and Lord *et al.*[9] showed that there was a change in the

infrared intensity of the C - D stretch in various solvents. Thus one might expect some interaction between the CH of chloroform and the C– Cl of carbon tetrachloride. There is, in fact, not only interaction between chloroform and carbon tetrachloride, but chloroform-chloroform interaction, as shown in TABLE 1. In addition, the amount of interaction is nearly the same as that found with the CH of methanol. We are thus forced to the conclusion that neither carbon tetrachloride nor chloroform is an "inert" solvent.

Since alcohol molecules may form hydrogen bonds with other types of molecules (for example, acetone) we have also examined these effects in solution and have found a pronounced effect on the OH absorption, as have others.[3a, 11] A single example is shown here, in FIGURE 9, of 0.1 molar methanol compared with a mixture of 0.1 molar methanol and 0.1 molar acetone. It is to be emphasized that the broken curve represents the absorption due to methanol in the mixture, assuming that the acetone absorption in this region is not affected by the methanol; that is, in computing this curve, the absorption of acetone in this region has been deducted from the absorption of the mixture. The curve indicates that, due to the acetone, both monomer and "polymer" reorient in such a manner as to increase the absorption in the 3500 cm.$^{-1}$ region; this implies the formation of a one-to-one acetone-alcohol complex.

The situation with intramolecular hydrogen bonding is considerably less clear. The literature contains mainly studies on acids, in which the effects are complicated by the presence of carboxyl groups. Kellner[4] states that intramolecular association "is easily distinguishable from intermolecular association by the fact that the band intensity does not change with concentration as

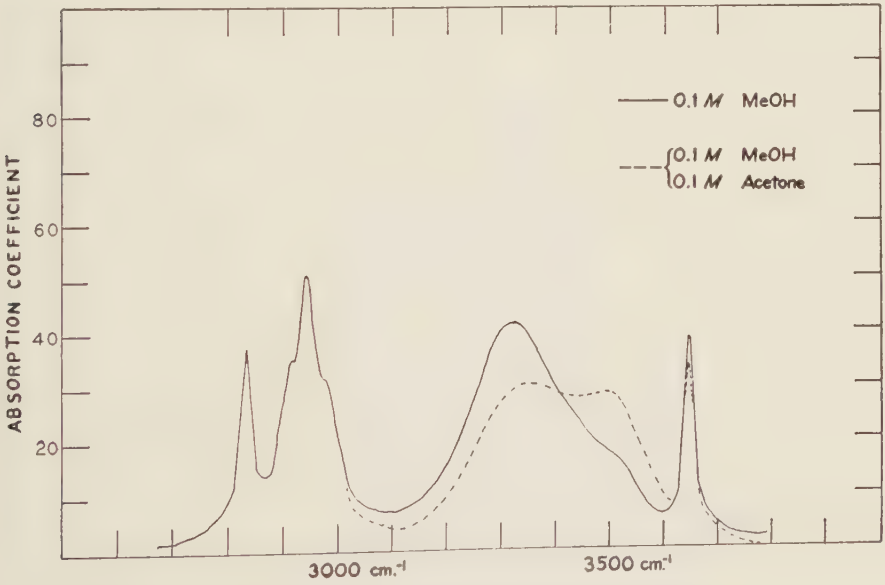


FIGURE 9. The effect of acetone on methanol absorption.

long as the product of cell length and concentration are kept constant . . . on the whole, the intramolecular association bands are much sharper than those found in intermolecular association." So few examples are given other than the carboxylic acids that it is difficult to assess the validity of these statements. Certainly neither is true in the case of salicylaldehyde and aldol. He further states that "on dilution of salicylaldehyde a weak monomeric band occurs indicating that a small proportion of the substance exists in the *trans* form." Without knowing how dilute these solutions were, it is difficult to comment. It has previously been pointed out[10] that salicylaldehyde sometimes contains small amounts of hydroxyl impurities, the presence of which might explain the statement.

Errera and Sack[11] studied a 10 per cent solution (by volume) of salicylaldehyde in carbon tetrachloride. They reported that salicylaldehyde "shows an absorption band, very broad but distinct with 2 maxima at 3130 80 cm.$^{-1}$ and at 3080." They go on to say, "This absorption is due to superposition of CH bands and a broad OH band, having a maximum at 3150 cm.$^{-1}$ Temperature variation does not influence the absorption." Tsubomura[12] gives 3180 cm.$^{-1}$ as $\tilde{\nu}_{max.}$ with a half width of 150 cm.$^{-1}$ and $\alpha_{max.}$ of 33 in our units.

We have studied pure salicylaldehyde and 0.1 molar and 0.01 molar solutions of salicylaldehyde in CCl_4. The spectrum of the 0.1 molar solution is given in FIGURE 10, together with the spectrum of 0.1 molar benzaldehyde for comparison. We are unable to agree with Errera and Sack that there are two

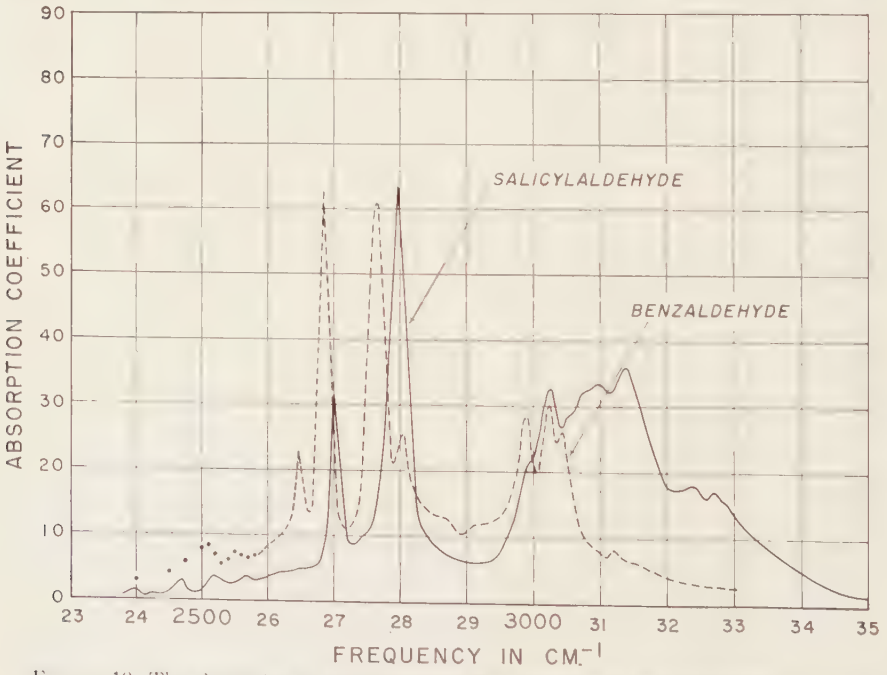


FIGURE 10. The absorption of salicylaldehyde (0.1 molar) and benzaldehyde (0.1 molar).

maxima. It seems to us that the whole region from 3000 to 3400 cm.$^{-1}$ is filled with a series of unresolved bands, completely unlike any known hydrogen-bonded spectrum previously presented. We have also examined the effect of temperature on the absorption of the dilute solution and find no apparent change between 0° and 60° C. Since Tsubomura regrettably did not show a spectrum we cannot make detailed comparison with his results.

The spectrum of the liquid sample is qualitatively the same as the spectra of the solution with regard to band structure. However, the ratio of the intensity of the 3000+ cm.$^{-1}$ absorption to the peaks at 2690 and 2790 increases from approximately 0.6 in the solution to nearly unity in the liquid. There must then be some concentration dependence, even though it is not observed in the range 0.01 to 0.1 molar.

Aldol (3-hydroxybutyraldehyde) seemed an interesting example of a molecule that might "bite its tail" by hydrogen bonding. The spectra do not lead to clean-cut interpretation. Since there can be intermolecular as well as intramolecular bonding, one might expect a complicated spectrum. Such is found. The spectrum as a function of concentration is shown in FIGURE 11. Temperature increase shows the same qualitative effect as decreasing concentration. One notes a dissimilarity to the alcohols. Now there are three bands in the associated region, one of which (3570 cm.$^{-1}$) does not appear to be strongly temperature- or concentration-dependent. The fact that the other two change so much in this low concentration range is unusual. The spectrum has some-

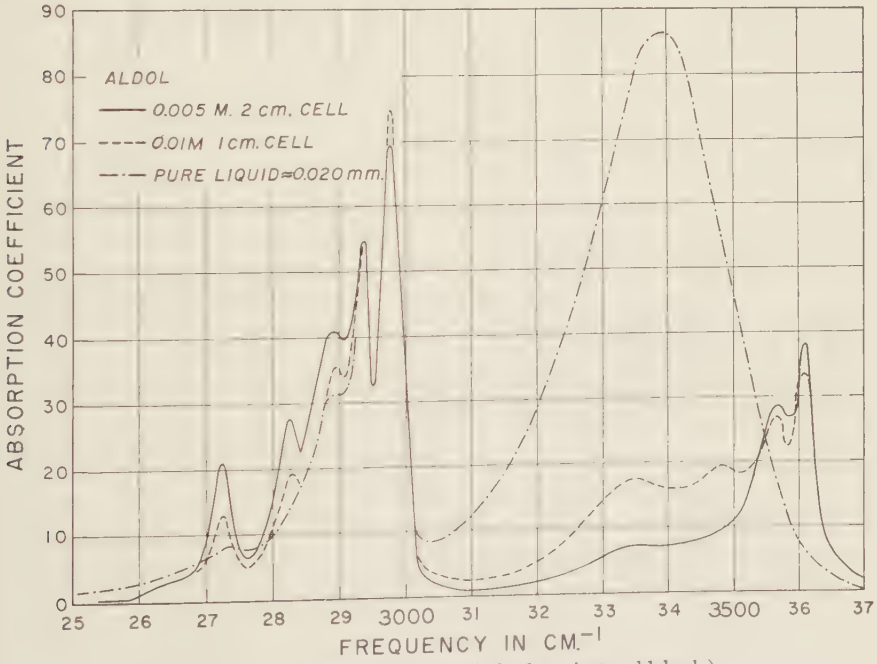


FIGURE 11. The absorption of aldol (β-hydroxybutyraldehyde).

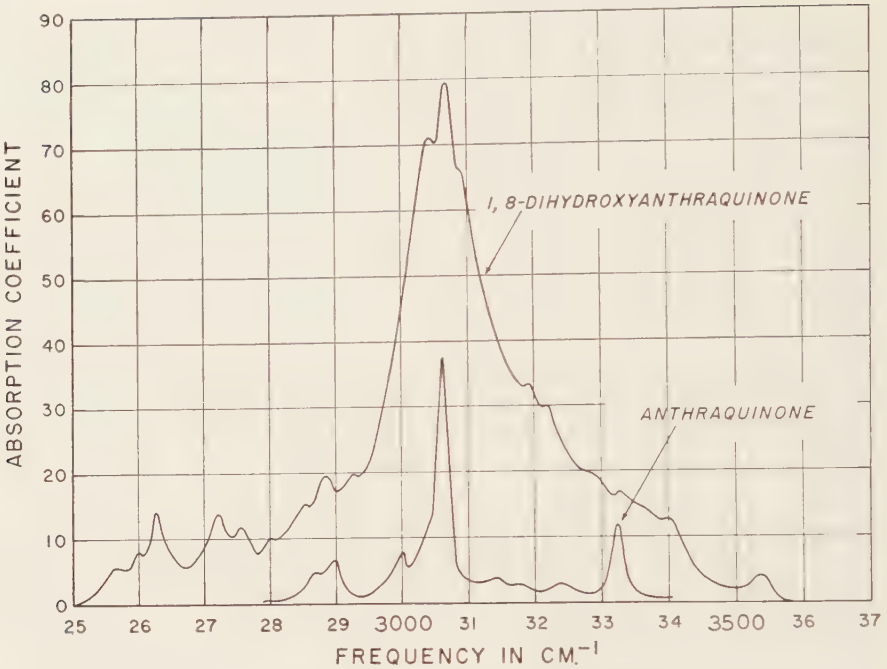


FIGURE 12. The absorption of anthraquinone and 1,8-dihydroxyanthraquinone.

what the appearance of the methanol-acetone mixture shown in FIGURE 9. In addition, the relatively large changes that occur in the bands from 2700 to 2900 cm.$^{-1}$ are noteworthy. If these do in fact originate from the CH portion of the molecule, it is the largest change of which we are aware. Since we have experienced such difficulty in purifying aldol and are uncertain as to the composition of our "pure" material, these results are presented largely to excite interest in this type of compound. Even if the material is entirely in a polymerized state, the spectrum is most unusual for a hydrogen-bonded compound.

A casual examination of the spectrum of 1,8-dihydroxyanthraquinone (FIGURE 12) would indicate that no absorption characteristic of the OH group is present. Comparison with the spectrum of anthraquinone in the same figure, however, shows that the absorption in the 3100 cm.$^{-1}$ region is many times as intense in the hydroxy compound as in the unsubstituted compound. It appears then that the two OH groups are bound to the same oxygen atom, and fortuitously absorb in the region usually ascribed to CH groups. The alternative explanation, that the intensities of the CH absorption are markedly increased by hydroxyl substitution in the molecule, has no counterpart in other spectra and is not considered plausible.

Bellamy,[13] in discussing this point, has assumed that the OH band is weak and is superimposed on a strong CH band. He was using the spectra of Flett,[14] which were taken with a spectrometer employing an NaCl prism. Flett also studied 1,2-dihydroxyanthraquinone and reported an OH band at 3380 cm.$^{-1}$

1,2-Dihydroxyanthraquinone (alizarin) presents an interesting example of a molecule that could have one OH bonded to the C=O and the second OH bonded to the first, according to the Pauling hypothesis. For this reason we included it in our study. We purified the material as described above and recorded the spectrum. It showed a broad complex in the 3100 cm.$^{-1}$ region and a sharp band at 3540 cm.$^{-1}$ We did not find any strong band at 3380 cm.$^{-1}$ as reported by Flett.[11] The concern over purity led us to have a sample prepared by recrystallization from glacial acetic acid. This material also had the correct melting point. A saturated solution in CCl_4 of this material ($\ll 0.002\ M$) showed only a band at 3540 cm.$^{-1}$ because of the low concentration. Consequently, we are not able to show a spectrum of alizarin at this time. It must suffice to say that apparently some normal OH absorption occurs and that it is intense compared with the CH absorption.

The spectrum of 1,4-dihydroxyanthraquinone (quinizarin) is shown in FIGURE 13. These data were computed on the basis that the solution was 0.0046 molar, although the concentration was uncertain, as noted above. This spectrum defies simple interpretation. The whole area is cluttered with unresolved bands. One might note that there is a small peak at 3530 cm.$^{-1}$ in the OH region, but there is no basis for assigning it to an OH vibration.

Lastly, as another example of the possibility of two hydroxyl groups bonding to the same oxygen, we examined 2,2′-dihydroxybenzophenone. The spectrum is shown in FIGURE 14. It exhibits the aspects of a normal intramolecular

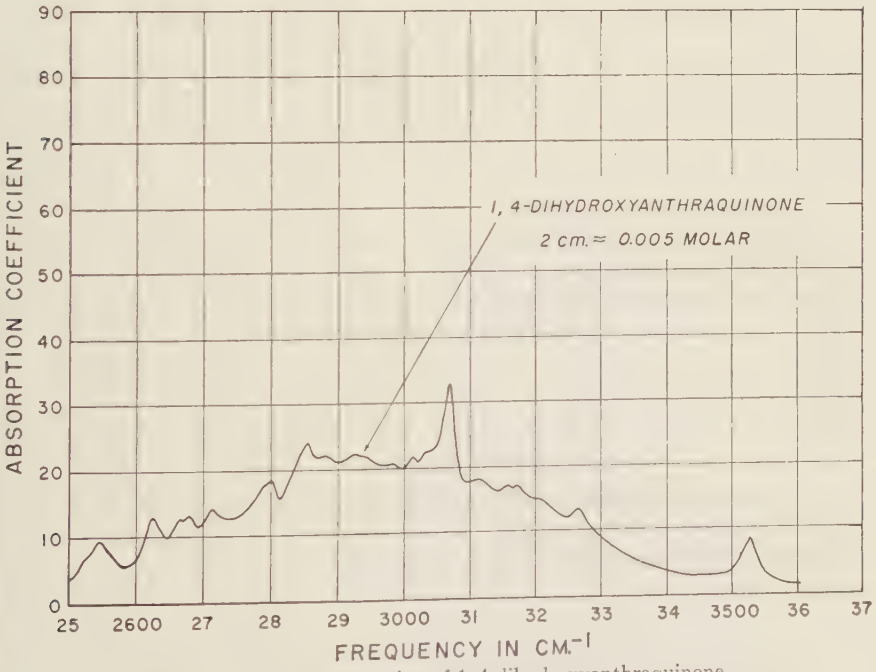


FIGURE 13. The absorption of 1,4-dihydroxyanthraquinone.

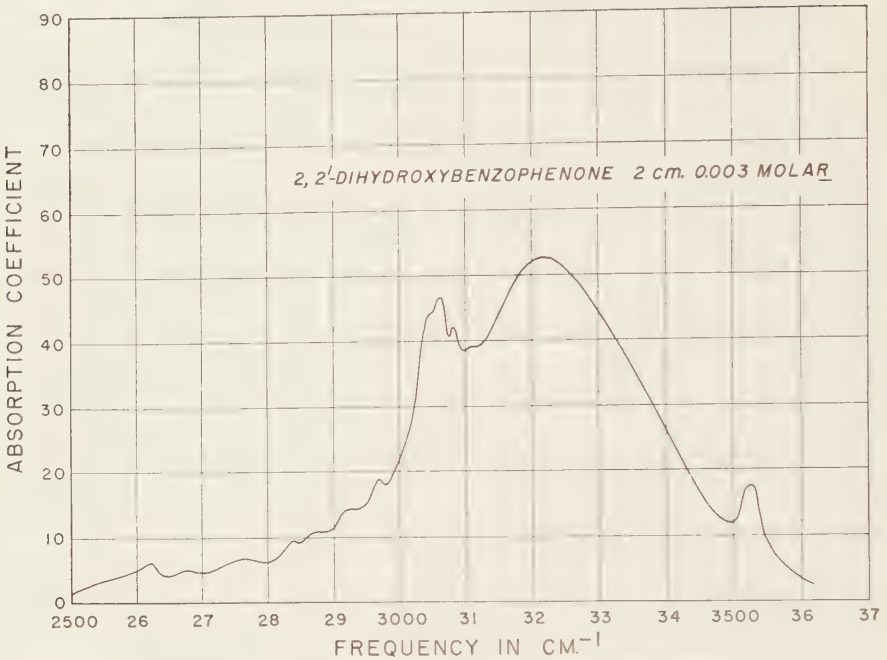


FIGURE 14. The absorption of 2,2′-dihydroxybenzophenone.

bonding, with a strong broad maximum at 3220 cm.$^{-1}$ Again there is a small maximum at 3525 cm.$^{-1}$ as in quinizarin, but much more work would be required to establish any relationship with an OH vibration.

It seems obvious from the foregoing that considerably more investigation must be carried out before we shall attain an understanding of intramolecular bonding and its effect on infrared spectra. The intermolecular association in the simpler alcohols now seems to be on as good a basis as possible with available techniques. The effect of adding other solutes seems straightforward. However, intramolecular bonding must be thoroughly examined before one can make the transition to interpretation of the spectra of the complex molecules active in biological systems. Such bonding is most important if we are to achieve a physical understanding of the forces between the atoms which are so significant in biological studies.

Acknowledgment

It is a pleasure to acknowledge the assistance of my colleagues, E. D. Becker, who did a considerable part of the experimental work on the alcohols, and N. E. Sharpless, who purified the anthraquinones.

References

1. HILBERT, G. E., O. R. WULF, S. B. HENDRICKS & U. LIDDEL. 1935. Nature. **135:** 147.
2. FREYMANN, R. 1933. Ann. Phys. **20:** 243.
3. ERRERA, J. & P. MOLLET. 1936. Nature. **138:** 882.

4. Kellner, L. 1952. Phys. Soc. Rept. Progr. in Phys. **15:** 1.
5. Brackett, F. S. 1957. J. Opt. Soc. Am. In press.
6. Van Thiel, M., E. D. Becker & G. C. Pimentel. 1957. J. Chem. Phys. In Press.
7. Coggeshall, N. D. & E. L. Saier. 1951. J. Am. Chem. Soc. **73:** 5414.
8a. Huggins, C. M., G. C. Pimentel & J. N. Shoolery. 1955. J. Chem. Phys. **23:** 1244.
8b. Huggins, C. M. & G. C. Pimentel. 1955. J. Chem. Phys. **23:** 896.
9. Lord, R. C., B. Nolin & H. D. Stidham. 1955. J. Am. Chem Soc. **77:** 1365.
10. Hilbert, G. E., O. R. Wulf, S. B. Hendricks & U. Liddel. 1936. J. Am. Chem. Soc. **58:** 549.
11. Errera, J. & H. Sack. 1938. Trans. Faraday Soc. **34:** 728.
12. Tsubomura, H. 1956. J. Chem. Phys. **24:** 927.
13. Bellamy, L. J. 1954. The Infrared Spectra of Complex Molecules. : 91. Wiley & Sons. New York, N. Y.
14. Flett, M. St. C. 1948. J. Chem. Soc. : 1441.

AQUEOUS SOLUTION INFRARED SPECTROSCOPY OF BIOCHEMICAL POLYMERS

By Elkan R. Blout

Chemical Research Laboratory, Polaroid Corporation, Cambridge, Mass., and Children's Cancer Research Foundation, Boston, Mass.

We live in an aqueous world and, because we do, it is apparent that the determination of the infrared spectra of materials of biochemical origin should be performed in aqueous solution—this being the "natural" environment of such materials. Furthermore, evidence is accumulating to show that the solvent environment markedly affects the configuration of naturally occurring compounds of high molecular weight, and that the configurational changes in such molecules play an important part in their biochemical functions. Investigation of the spectra of proteins, nucleic acids, and polypeptides in aqueous solution permits the examination of changes in molecular configuration induced by temperature, denaturing agents, and enzymatic reactions. In this paper an attempt will be made to show how the infrared spectra of certain biochemical compounds in aqueous solution can be used to give evidence of both chemical and molecular-configurational changes.

Because most spectroscopists know that water is a strong absorber of infrared radiation, only recently has water been used as a solvent in infrared spectroscopy. The first report on its use appears to be that of Gore, Barnes, and Petersen[1] who, in 1949, reported the infrared absorption spectra of aqueous solutions of organic acids. These workers appreciated the fact that, although water was strongly absorbing in some of the infrared, in other parts it was relatively transparent, especially when thin layers were used. They further realized that deuterium oxide was transparent where water absorbed, and vice versa, so that by the combined use of water and deuterium oxide solutions it was possible to cover the spectral region from 700 to 4000 cm.$^{-1}$

FIGURE 1[2] shows the transmittance of water and heavy water in a thickness of 0.025 mm. in the region 600 to 4000 cm.$^{-1}$ The most prominent absorption bands are the strong O—H deformation modes of water between 1600 and 1700 cm.$^{-1}$ and the corresponding O—D deformations in heavy water around 1200 cm.$^{-1}$ Except for these two regions, both spectra show greater than 40 per cent transmittance, so that water and deuterium oxide can be used as solvents, especially in double-beam instruments. The balance obtainable with 0.025 mm. of D_2O in each beam of a double-beam spectrometer (with reasonable response and resolution) is shown in the top curve. It is, of course, well known that the use of a double-beam spectrometer makes possible the spectral determination of materials having higher absorption than can be determined conveniently in single-beam systems. Thus, from inspection of the curves shown in FIGURE 1 it is manifestly possible (using a double-beam spectrometer) to obtain good spectral data on water-soluble compounds, provided they have sufficient solubility so that a 0.025 mm. layer gives reasonable absorption. We have found by experience that a compound must be soluble to the extent of at least 2 per cent by weight if we are to obtain satisfactory infrared spectral data in

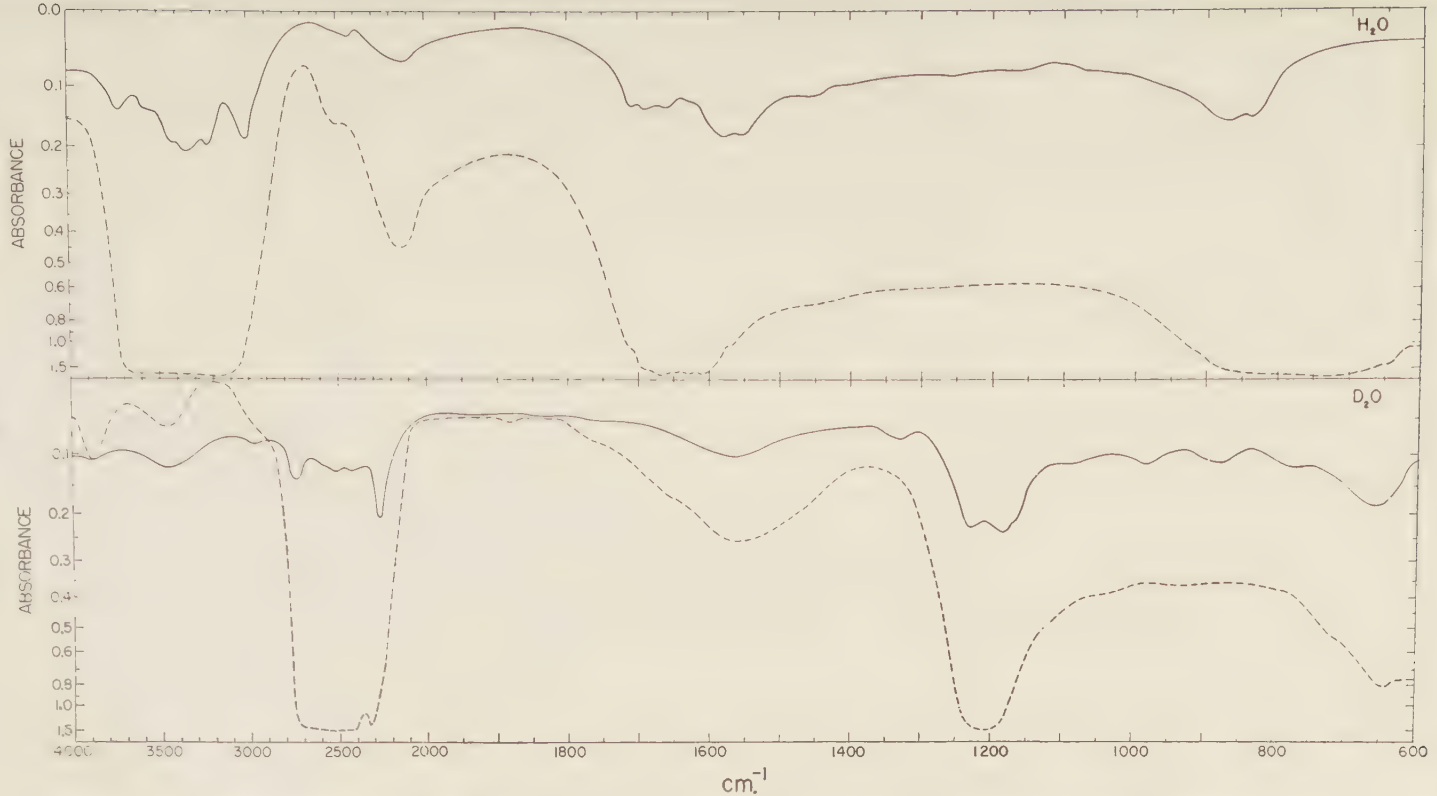


FIGURE 1. The infrared spectra of H_2O and D_2O. – – – – Spectra with no compensation in the reference beam. Thickness 0.025 mm. —— Spectra showing balance obtainable in a double-beam spectrometer using 0.025 mm. of the appropriate solvent in each beam.

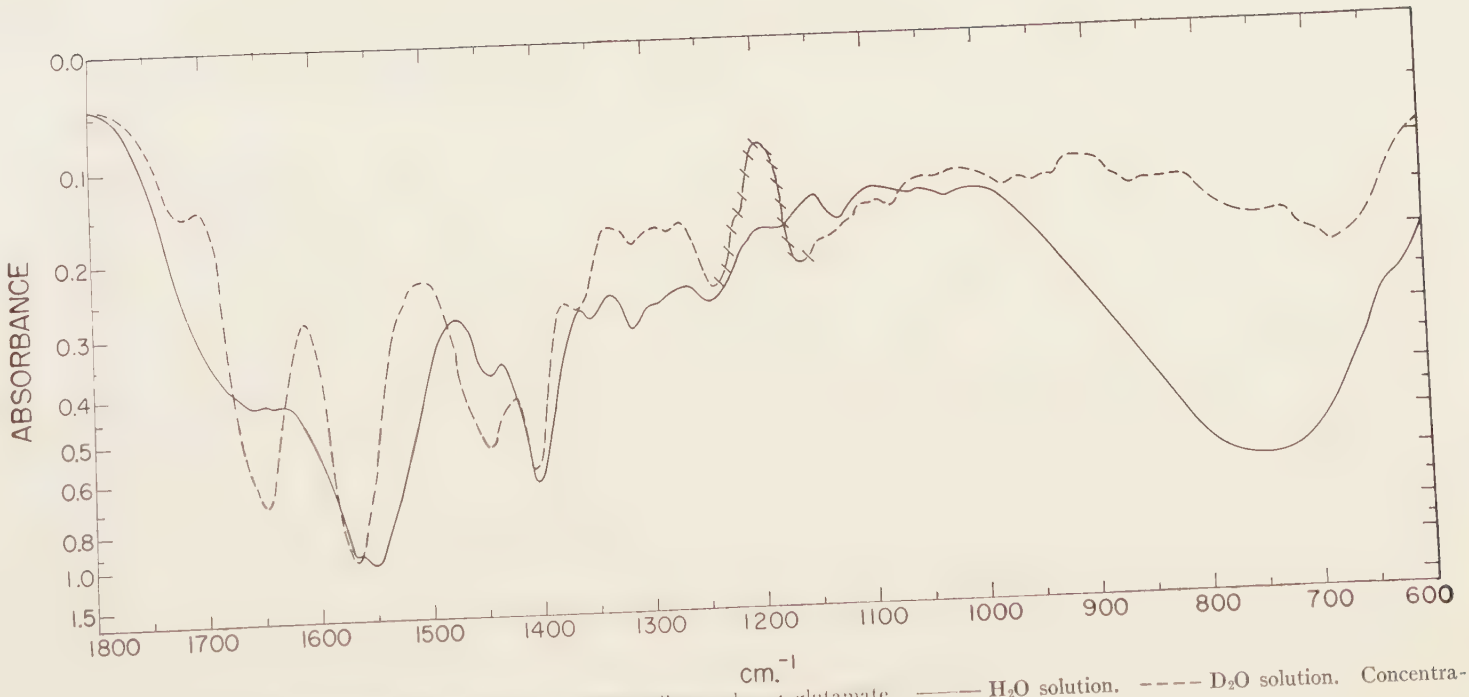


FIGURE 2. The infrared spectrum of high molecular weight sodium poly α L glutamate. —— H_2O solution. - - - - D_2O solution. Concentration: 5 per cent by weight. Thickness: 0.025 mm. Solvent compensated.

aqueous solutions. Actually, the optimum concentration of solute lies in the range of 5 to 20 per cent, the same concentration as that of many biological polymers, such as proteins, in cells and tissues.

Having concluded that it is possible to obtain spectral data on aqueous solutions, we next inquire: for what type of compound would such data be of particular interest? It is our opinion that, aside from the obvious utility of infrared spectra for establishing identity of biochemical substances, the most promising investigative field is the use of infrared spectroscopy to determine changes of position, shape, or configuration of such biologically important high polymers as synthetic polypeptides, proteins, nucleic acids, and nucleoproteins. The remaining part of this paper will be concerned with specific examples of infrared spectroscopy of such compounds in aqueous solution.

Polypeptides

The infrared spectra of a water-soluble high-molecular-weight polypeptide, sodium poly-α-L-glutamate, are reproduced in FIGURE 2. The solid line shows the spectrum in H_2O solution and the dashed line the spectrum in D_2O solution. Several absorption bands can be correlated with specific chemical groupings.

First, the band at 1650 cm.$^{-1}$ is the carbonyl amide (or amide I) band typical of polypeptides and proteins. It is very much weaker in the spectrum of the H_2O solution than in the spectrum of the D_2O solution. The band is not actually weaker in water solution, but water is so strongly absorbing in this region (FIGURE 1) that the spectrometer shows essentially no response here.

The second absorption region of interest lies between 1500 and 1600 cm.$^{-1}$ There is a doublet at 1575 and 1550 cm.$^{-1}$ in the H_2O spectrum, whereas in the D_2O spectrum only one band appears, namely that at 1575 cm.$^{-1}$ The reason for this behavior is that the 1575 cm.$^{-1}$ band has been shown to be due to ionized carboxyl,[3] the frequency of which should not be much affected by its environment; on the other hand, the band at 1550 cm.$^{-1}$ (called amide II and characteristic of polypeptides and proteins) involves an NH deformation coupled with C=N stretching modes of the peptide group.[5] Treatment with D_2O of compounds containing "active" hydrogen, such as amines or amides, results in at least some replacement by deuterium of the hydrogen attached to the nitrogen. Since the vibrational mass is increased when deuteration occurs, there is a lowering of the absorption frequency in this case from 1550 cm.$^{-1}$ to approximately 1450 cm.$^{-1}$ In the sodium poly-α-L-glutamate spectrum shown in FIGURE 2 the secondary amide hydrogens have been replaced by deuterium and the band at 1550 cm.$^{-1}$ is not observed at all in the D_2O spectrum, but the rather strong band at 1450 cm.$^{-1}$, due to the deuterated amide II absorption, is noted.

The remaining parts of the spectra of the D_2O and H_2O solutions are essentially similar, except that there is some uncertainty (indicated by the cross-hatched lines) between 1150 and 1250 cm.$^{-1}$ in the D_2O spectrum due to the absorption of the D_2O in this region and to the slight difference in thickness of the solution cells. Similarly, there is an absorption peak around 750 cm.$^{-1}$ in the spectrum of the water solution, again due to the difference in path length and to the strong absorption by H_2O in this region.

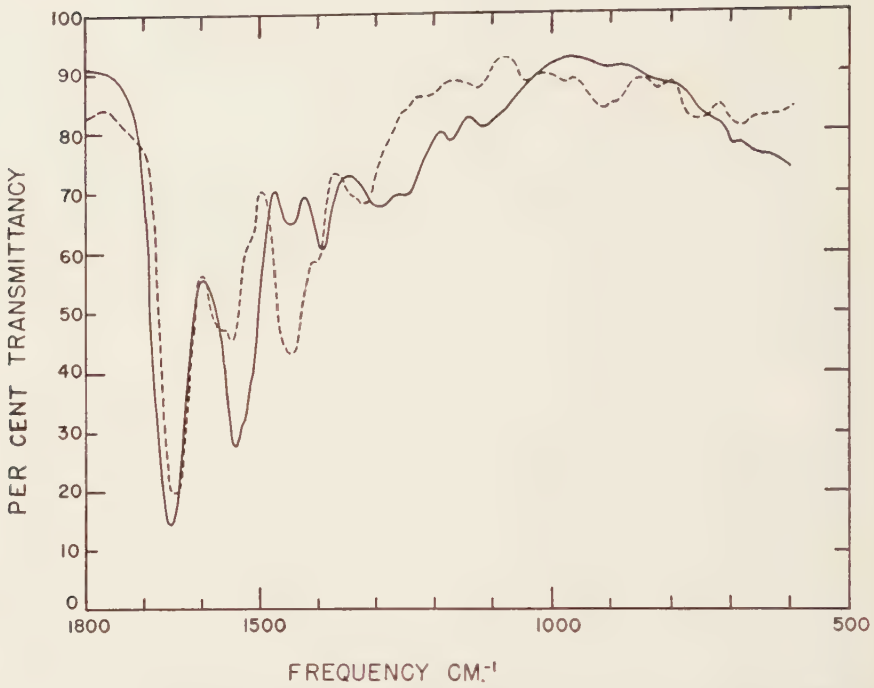


FIGURE 3. The infrared spectra of bovine plasma albumin. ——— A dry film cast from water on a silver chloride plate. – – – – A 10 per cent solution in D_2O. Solvent compensated. (Reproduced by permission of the *Journal of the Optical Society of America*.[2])

If the spectrum of poly-α-L-glutamic acid in D_2O:dioxane solution[6] is compared with that of sodium poly-α-L-glutamate (FIGURE 2), it is seen that as the *p*D* is lowered there is increasing absorption intensity in the 1710 cm.$^{-1}$ (–COOD) band and decreasing intensity observed in the 1575 cm.$^{-1}$ ($COO^{=}$) band. This suggests that it should be possible to measure ionization, or possibly even carboxyl content, of high polymers at various *p*D's using aqueous-solution infrared spectra. Moreover, as the *p*D is increased in D_2O solutions of poly-α-L-glutamic acid the frequency of the carbonyl (amide I) absorption shows a shift toward higher frequencies. This change in frequency may be attributed to changes from a helical configuration to nonhelical configurations of the backbone polypeptide chain.[6]

Proteins

The infrared spectra of bovine plasma albumin as a dry film (cast from water on a silver chloride plate) and in solution in heavy water are seen in FIGURE 3. In the D_2O solution spectrum there is a shift of the amide II frequency from 1550 to 1450 cm.$^{-1}$ due to its deuteration, and a slight shift in the amide I frequency from approximately 1655 to 1645 cm.$^{-1}$ The latter absorption band

* The term *p*D is employed (rather than *p*H) to indicate the deuterium ion concentration in the D_2O solutions used in these investigations.

is due (primarily) to a carbonyl stretching vibration, which is hydrogen-bonded in the case of the dry film, whereas in the D_2O solution it is deuterium-bonded. For the reasons mentioned above its frequency is lowered slightly when it is deuterium-bonded. Also, it should be noted that in the heavy-water solution there is a band at 1575 cm.$^{-1}$ (ionized carboxyl) that appears as a strong shoulder, but is not seen at all in the spectrum of the dry film.

Recently we have examined the effect of alteration of *p*D on the infrared spectrum of bovine serum albumin in D_2O solution.[4] Two things may be noted: first, at *p*D 3 there is no evidence of a band at 1575 cm.$^{-1}$; second, at *p*D 3 there is a band at 1550 cm.$^{-1}$ (amide II) that may be ascribed to undeuterated amide groups. At *p*D 4.5 there is evidently some ionized carboxyl, as the 1575 cm.$^{-1}$ band begins to appear. At the isoelectric point, around *p*D 5, there is increased absorption in the ionized carboxyl band and still an undeuterated amide II band at 1550 cm.$^{-1}$ If the *p*D is raised to 8, the 1550 cm.$^{-1}$ band is completely removed. This suggests that in bovine serum albumin there are some strong bonds, perhaps hydrogen bonds, which are so strong in the native configurations of proteins that at *p*D's below or close to the isoelectric point they do not allow the penetration of solvent D_2O into the structure. It is only at high *p*D's that these structures are broken down or opened sufficiently to allow the penetration of the D_2O molecules and hence the exchange of deuterium for amide hydrogen. Since it is known that α-helical structures[7] exist in synthetic polypeptides and are strongly intramolecularly hydrogen-bonded, it is not unwarranted to assume that such structures exist in part in native proteins. Therefore, if the hard-to-deuterate amide were to correspond to the helical portion of protein molecules, then by measuring the infrared spectra of proteins at various *p*D's, one might be able to determine the content of helical structures of the proteins.

We have observed, also,[4] that when bovine serum albumin is denatured by heat, with the possible result that the helical portion of the protein is destroyed, there is ready exchange (in heavy water) of the amide groups at all *p*D's, as attested by the lack of a 1550 cm.$^{-1}$ absorption band at any *p*D.

The infrared spectra of several different proteins at their isoelectric points in D_2O solution have been determined.[4] It was observed that the intensity of the 1575 cm.$^{-1}$ band (ionized carboxyl) varies with the protein. Consequently some measurements were made to ascertain whether the carboxyl content of proteins might be determined by measuring the intensity of this 1575 cm.$^{-1}$ band in D_2O solution. The data for serum albumin, ovalbumin, and γ-globulin are shown in TABLE 1. It can be seen that there is an approximate correlation between the carboxyl content obtained from infrared spectra and that from titration.

Nucleic Acids

It has been shown[9a, 9b] that deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have characteristic infrared spectra when measured as solid-state samples. These spectra allow differentiation between the two types of nucleic acids, principally by means of a medium-intensity band at 1020 cm.$^{-1}$ that is present in deoxyribonucleic acid and absent in ribonucleic acid. A similar

TABLE 1

	Infrared data*	Titration data†
	Absorbance ratio $\frac{1575\ cm.^{-1}\ band}{1650\ cm.^{-1}\ band}$	Free carboxyls‡
Serum albumin	0.54	124
Ovalbumin	0.44	98
γ-Globulin	0.34	67

* Based on D_2O solution at *p*D 8; compensated.
† From Haurowitz.[8]
‡ Moles amino acid per 10^5 gm. protein.

situation exists in D_2O solution;[2] one can differentiate the two types of nucleic acid by means of a band near 1020 cm.$^{-1}$, although in the solution spectra the band has shifted to a somewhat higher frequency and lies at 1060 cm.$^{-1}$

It is relevant now to inquire what other information can be obtained from aqueous-solution spectra of nucleic acids. In attempting to answer this question we have used DNA in D_2O solution and made studies of the following: (1) the effect of changing *p*D; (2) the effect of changing temperature; and (3) the effect of deoxyribonuclease on the infrared spectrum of DNA. Portions of these studies have been published[10a, 10b] and will only be summarized here. The only spectral region considered here is that between 1550 and 1750 cm.$^{-1}$ This is the region of strong absorption by DNA. The several bands seen in this region are due to C=O stretching motions, to C=N stretching motions, and to ring frequencies of the purine and pyrimidine ring moieties of deoxyribonucleic acid.

Low-molecular-weight DNA shows a strong absorption band at 1660 cm.$^{-1}$ that remains essentially unchanged as the *p*D of the solution is raised from 2 to 11. However, two weaker bands at 1625 cm.$^{-1}$ and 1575 cm.$^{-1}$ show increasing intensity with increased *p*D. High-molecular-weight, or native, DNA shows its strongest absorption band in this region at 1680 cm.$^{-1}$ at *p*D's from 5 to 9, with a less intense band at about 1645 cm.$^{-1}$ However, as the *p*D of the native DNA solution is raised to 11, the 1680 cm.$^{-1}$ band shifts to 1660 and the 1645 band shifts to 1625 cm.$^{-1}$ If the *p*D of the solution is then returned to *p*D 6, there is no indication of the return of the 1660 and 1625 bands to their original frequencies, namely 1680 and 1645 cm.$^{-1}$ This can be interpreted as indicating that an irreversible change in the structure of the DNA molecule has occurred. Since the observed spectral change occurs in the region known to be associated with hydrogen- or deuterium-bonded groups, it appears that the change has affected this bonding.

We report here the effect of varying the temperature of a solution of DNA in D_2O at *p*D 6 (measured at 25° C.). The spectral changes are seen in FIGURE 4. The characteristic spectrum of native DNA is shown in the top curve. There is little change in the spectrum between 25° and 64° C. However, when the temperature is raised to 92° C., the 1680 band shifts to 1660 cm.$^{-1}$, and the 1645 band shifts to 1625 cm.$^{-1}$ If now the solution is cooled quickly to 25° C.,

essentially the original (native DNA) spectrum is obtained, namely that with the bands at 1680 and 1645 cm.$^{-1}$. These data can be interpreted as meaning that the changes brought about by heat in this experiment are reversible ones, in contrast to the irreversible ones (reported above) induced by high *p*H or *p*D. It appears that heating DNA solutions to about 90° C. results in a breakdown of the helical hydrogen-bonded structure of this molecule,[11, 12] but without causing complete loss of configurational integrity, so that its original configuration can be reformed upon cooling. We interpret the infrared results as

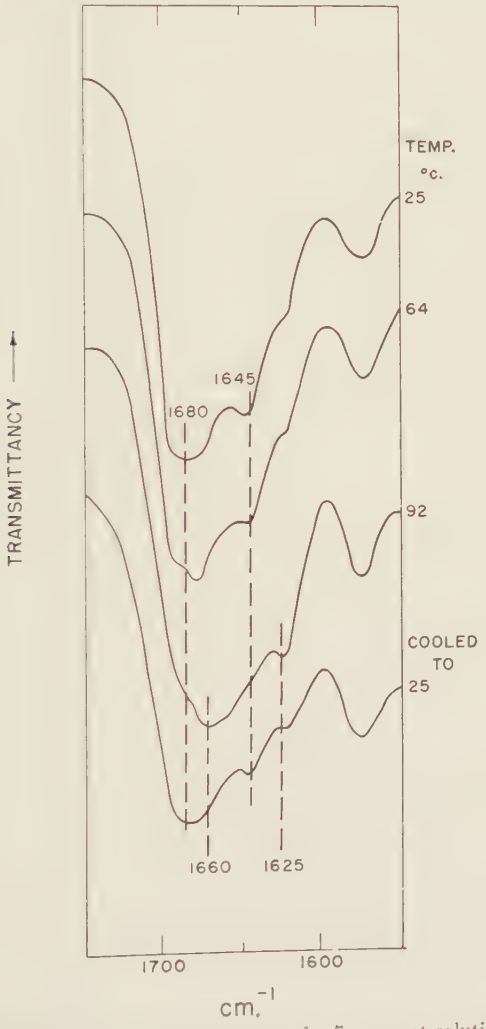


FIGURE 4. A portion of the infrared spectrum of a 7 per cent solution of sodium deoxyribonucleate in D_2O. The numbers at the right are the solution temperatures (in °C.) at which the spectral measurements were made. Solvent compensated.

meaning that the 1680 and 1645 cm.$^{-1}$ absorption bands are characteristic of the helical structure of native DNA, whereas the 1660 and 1625 cm.$^{-1}$ bands are characteristic of nonhelical or denatured forms of this molecule.

Changes due to enzyme action can be followed in certain cases through infrared spectral determinations of nucleic acids and proteins in aqueous solutions. For example, the addition of a trace of deoxyribonuclease to a gel of high-molecular-weight DNA at 37° C. liquefies the gel completely in $2^1{}_2$ hours. Concomitant with the liquefaction of the gel, the infrared spectrum changes markedly: the 1680 and 1645 cm.$^{-1}$ bands disappear and the bands at 1660 and 1625 cm.$^{-1}$ appear; as mentioned above, the appearance of the latter bands indicates a breakdown of the native helical configuration of DNA.

Nucleoproteins

The infrared spectra of tobacco mosaic virus (TMV) in D_2O solution has also been investigated.[13] This material, prepared by Norman Simmons, is a nucleoprotein with molecular weight of about 50,000,000 in its native state. It shows a typical nucleoprotein spectrum, but for our present purpose we shall concentrate our attention on the region 1500 to 1700 cm.$^{-1}$ (FIGURE 5). We have noted above that in D_2O solutions of proteins the amide I, or C═O, frequency is located at about 1645 cm.$^{-1}$ Since tobacco mosaic virus is about 95 per cent protein, it is not surprising that this frequency is very strong in its

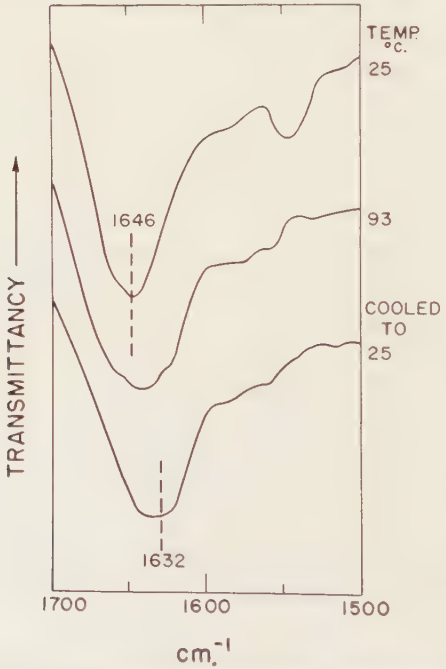


FIGURE 5. A portion of the infrared spectrum of a 7 per cent solution of tobacco mosaic virus in D_2O. The numbers at the right are the solution temperatures (in °C.) at which the spectral measurements were made. Solvent compensated.

infrared spectra. On raising the temperature of a 7 per cent solution of TMV in D_2O, the normal amide I frequency at 1646 cm.$^{-1}$ gradually shifts to lower values and finally appears at 1632 cm.$^{-1}$ It might be mentioned parenthetically that the latter frequency is characteristic of the undeuterated amide I group when the chain is in an extended or β configuration in the solid state.[14, 15] If the D_2O solution of TMV is cooled to 25° C., the amide I frequency does not return to its original position, indicating that, in contrast to the DNA solution, the change induced in the TMV molecule is an irreversible one.

Conclusions

It is hoped that the foregoing indicates not only that it is possible to obtain infrared spectra of aqueous solutions of high-molecular-weight naturally occurring biological polymers, but also that from such systems can be obtained information that is not easy to acquire using other physical techniques. It appears that by applying infrared spectroscopy to such materials in aqueous solutions (both D_2O and H_2O) it is possible to follow enzymatic reactions and, possibly, other metabolic processes. In addition, there appears to be little doubt that this method is important for the study of configurational changes in such high-molecular-weight compounds, especially with respect to hydrogen and deuterium bonding.

Acknowledgment

It is a pleasure to acknowledge our gratitude to our long-time collaborator in much of this work, Henri Lenormant of the Laboratoire de Physiologie Générale de la Sorbonne, Paris. We also express our thanks to Anna Asadourian for her technical assistance, and to the Office of the Surgeon General, Department of the Army, Washington, D. C., whose generous support of the polypeptide portion of this work has made it possible.

References

1. Gore, R. C., R. B. Barnes & E. Petersen. 1949. Anal. Chem. **21:** 382.
2. Blout, E. R. & H. Lenormant. 1953. J. Opt. Soc. Am. **43:** 1093.
3. Lenormant, H. & E. R. Blout. 1953. Nature. **172:** 770.
4. Lenormant, H. & E. R. Blout. 1954. Bull. soc. chim. France. : 859.
5. Fraser, R. D. B. & W. C. Price. 1952. Nature. **170:** 490.
6. Doty, P., J. T. Yang, A. Wada & E. R. Blout. 1957. J. Polymer Sci. **23:** 851.
7. Pauling, L. & R. B. Corey. 1951. Proc. Natl. Acad. Sci. U. S. **37:** 241.
8. Haurowitz, F. 1950. Chemistry and Biology of Proteins. : 32. Academic Press. New York, N. Y.
9a. Blout, E. R. & M. Fields. 1948. Science. **107:** 252.
9b. Blout, E. R. & M. Fields. 1949. J. Biol. Chem. **178:** 335.
10a. Blout, E. R. & H. Lenormant. 1954. Biochim. et Biophys. Acta. **15:** 303.
10b. Blout, E. R. & H. Lenormant. 1955. Biochim. et Biophys. Acta. **17:** 325.
11. Watson, J. D. & F. H. C. Crick. 1953. Nature. **171:** 737.
12. Crick, F. H. C. & J. D. Watson. 1954. Proc. Roy. Soc. London. **A223:** 80.
13. Simmons, N. & E. R. Blout. Unpublished results.
14. Ambrose, E. J. & A. Elliott. 1951. Proc. Roy. Soc. London. **A205:** 47.
15. Blout, E. R. & M. Idelson. 1956. J. Am. Chem. Soc. **78:** 497.

THE SOLID-STATE INFRARED ABSORPTION OF THE OPTICALLY ACTIVE AND RACEMIC STRAIGHT-CHAIN α-AMINO ACIDS

By Robert J. Koegel, Rita A. McCallum, Jesse P. Greenstein, Milton Winitz, and Sanford M. Birnbaum

Laboratory of Biochemistry, National Cancer Institute, Public Health Service, Bethesda, Md.

The volume of current literature dealing with the application of solid-state infrared absorption spectroscopy to the solution of quantitative, qualitative, and structural problems in the biological and biochemical fields is ample evidence of the increasing interest and broadening usefulness of this technique. The literature has been summarized in several current reviews.[1-4]

Implicit in each of these reviews and in every application of infrared absorption spectroscopy to biological problems—particularly, in those that are to be made quantitative or are concerned with structural interpretation—are certain general principles that govern the correlation of the vibrational spectra of large molecules with their structural features. These are: (1) each specific configuration of atoms forming a molecule will exhibit a unique and characteristic vibrational spectrum; (2) this spectrum is a function of the masses of the constituent atoms and the forces that maintain the equilibrium spatial configuration of the molecule; and (3) although each observed absorption band reflects to some extent motions of the entire molecule, it has been conclusively established that many of the absorption bands in the spectra of complex molecules are to a considerable degree determined by the vibrational characteristics of isolated functional groups within the structure. As it has only been possible to solve the classical equations of motion for relatively simple molecules possessing a high degree of symmetry, the approximation of a vibration localized in a single bond is basic to the application of infrared spectroscopy to biological problems and, in particular, to those concerned with determining molecular structures. It is well recognized that the continued and ever-widening application of infrared techniques is to a very large degree directly dependent upon increasing the accuracy with which adequately resolved, sufficiently intense absorption bands can be correlated with specific molecular vibrations, and that this increased accuracy will be realized only when all the factors affecting the constancy of characteristic group frequencies are completely understood.[6, 7]

Very early in the application of solid-state infrared absorption spectroscopy to molecules of biochemical interest it became clear that the presence of asymmetric carbon atoms influenced the constancy of characteristic group frequencies.[8, 9] It was observed that the infrared spectra of optically enantiomorphic forms were alike, but differed in every case from that of the corresponding racemic compound. These differences have not always been taken into consideration. This paper is concerned with a characterization of the spectra of a homologous series of L-amino acids whose optical purity is greater than 99.9 per cent (Sutherland[9]), and their DL-counterparts, and with an attempt to extend the assignment of reliable group frequencies.[10, 11]

Infrared Absorption Technique

The spectra that comprise the data of this paper were recorded with a Perkin-Elmer Model No. 21 double-beam infrared spectrometer equipped with sodium chloride optics and calibrated against the known absorption bands of ammonia, polystyrene, and atmospheric H_2O. The solid-state spectra were taken using the potassium bromide pellet technique.[12,13] The liquid-phase spectra were obtained on D_2O (Stuart Oxygen Co., D_2O 99.5 per cent) solutions, using CaF_2 cells of approximately 0.01 mm. D_2O has a strong absorption band near 1538 cm.$^{-1}$ (6.50 μ) that may correspond to the Raman line at approximately 1600 cm.$^{-1}$ and has been attributed to a hindered rotation of deuterium-bonded water molecules around the O–D axis; however, the energy levels reaching the receiver are more than adequate, and, with proper compensation in the reference beam, spectra may be conveniently recorded from 1905 cm.$^{-1}$ (5.25 μ) to 1274 cm.$^{-1}$ (7.85 μ).[14] All spectra were recorded using control settings recommended by Perkin-Elmer for reference spectra; in order to realize fully the resolution available with this slit schedule, the spectra were scanned at a rate of 5 min. per micron.

Source of the Straight-Chain Optically Enantiomorphic α-Amino Acids

The synthesis and resolution of the chemically and optically pure model structures used in this study has been described fully.[15-17] Each straight-chain α-amino acid is chemically pure, and the L-forms are optically pure in excess of 99.9 per cent.[14-16]

Solid-State Infrared Absorption of a Homologous Series of L-α-Amino Acids

The first systematic study of the vibrational spectra of α-amino acids was made by Edsall and his co-workers.[18-24] This early work was concerned primarily with Raman spectra in aqueous solutions, and it furnished strong corroborative data for the dipolar structure of the α-amino acids. The corresponding usefulness of aqueous-phase infrared spectra was demonstrated by Gore *et al.*,[25] who investigated the absorption of glycine in deuterium oxide. Lacher *et al.*[26] used antimony trichloride as a solvent for infrared absorption studies on the strongly polar α-amino acids. Solution spectra have furnished many significant and valuable data characterizing the absorption of amino acids; however, the technique is both technically more difficult and more limited in application than solid-state spectra. Therefore, solid-state techniques have been used in most of the studies that have been done on infrared absorption of amino acids. Such studies have been reviewed by Sutherland,[1] and the solid-state absorptions of about fifty pairs of optically enantiomorphic α-amino acids have been recently characterized by Koegel *et al.*[10]

The 4000 to 1650 cm.$^{-1}$ region. In TABLE 1 are listed all the significant solid-state absorption bands from 5000 to 667 cm.$^{-1}$ of the straight-chain L-amino acids studied. Each absorption peak is given a numerical value (% A) that expresses in a semiquantitative way its peak intensity. This numerical measure of intensity permits a comparison of the absorption of a specific peak with

Glycine																			
λ	3.23	3.45	3.57		3.90	4.77		6.25			6.60		6.93	7.10			7.52		
Desc.	S	B	B		S	B		VB			VB		VS	B			VS		
% A	51	52	56		55	23		69			68		53	67			65		
L-Alanine																			
λ	3.29	3.40	3.55	3.73	3.90	4.75	6.16	6.28		6.55		6.87		7.08	7.38			7.65	
Desc.	S	sh	B	B	S	B	S	S		VS		VS		VS	VS			VS	
% A	43		37	35	31	9	65	78		25		31		47	62			60	
L-α-Amino-*n*-butyric acid																			
λ	3.32	3.43			3.85	4.73		6.23	6.33		6.64	6.83	6.92	7.13	7.38	7.53	7.57	7.67	
Desc.	sh	B			sh	B		sh	B		S	VS	S	S	VS	sh	VS	VS	
% A		80				14			95		92	63	54	88	54		68	41	
L-Norvaline																			
λ	3.35	3.47	3.55		3.86	4.70		6.23	6.33		6.66	6.82	6.92	7.10	7.38		7.58		7.
Desc.	sh	S	sh		sh	B		sh	B		B	VS	S	S	S		S		V
% A		68				10			80		74	51	55	74	59		56		4
L-Norleucine																			
λ	3.32	3.43	3.57		3.88	4.70		6.27	6.33		6.60	6.84	6.91	7.14	7.38		7.57	7.67	
Desc.	sh	S	sh		S	B		sh	VB		B	S	B	S	S		VS	sh	
% A		68			36	24			70		68	40	45	67	46		60		
L-α-Amino-*n*-heptylic acid																			
λ	3.28	3.43	3.52		3.87	4.70		6.20	6.30	6.57	6.82	6.90		7.07	7.36		7.55		7.
Desc.	sh	VB	sh		sh	B		sh	S	S	sh	VS		S	VS		S		
% A		56				17			67	63		56		59	42		48		1
L-α-Amino-*n*-octanoic acid																			
λ	3.33	3.47	3.57			4.68		6.20	6.31		6.60	6.81	6.92	7.10	7.36		7.57	7.79	7.
Desc.	sh	VB	sh			VB		sh	S		S	S	VS	S	VS		VS	S	
% A		56				14			64		60	37	49	53	36		45	15	1
L-α-Amino *n* nonylic acid																			
λ	3.34	3.47	3.52		3.90	4.70		6.23	6.33		6.60	6.83	6.93	7.10	7.36		7.57		7.
Desc.	sh	VB	B		sh	B		sh	B		B	B	VS	S	S		VS		
% A		46	42			9			60		55	35	44	49	25		42		
L-α-Amino-*n*-decylic acid																			
λ	3.35	3.48	3.57			4.73		6.20	6.37		6.61	6.82	6.93	7.13	7.37		7.57	7.87	7
Desc.	sh	VB	sh			B		sh	VB		S	VS	VS	S	VS		S	S	
% A		40				10			67		61	45	54	58	38		48	7	
L-α-Amino-*n*-undecylic acid																			
λ	3.37	3.50	3.56			4.74		6.26	6.35		6.61	6.83	6.94	7.10	7.37		7.57	7.88	7
Desc.	sh	B	sh			VB		sh	S		S	S	VS	S	VS		VS	S	
% A		85				10			90		82	41	62	74	40		62	7	
L-α-Amino-*n*-dodecylic acid																			
λ	3.37	3.48	3.57			4.78		6.23	6.32		6.63	6.83	6.92	7.09	7.43	7.47		7.77	7
Desc.	sh	B	B			VB		sh	B		VS	VS	sh	VS	VS	VS		VS	
% A		44	43			10			84		49	43		72	60	57		25	

% A = Per cent absorption; B = broad; S = strong; sh = shoulder; VB = very broad; VS = very strong.

	8.85 S 29	9.02 B 35			9.68 B 28				10.98 VS 46	11.21 VS 51									14.37 VB 41	
70 S 4	8.98 VS 44					9.83 S 22			10.88 S 12			11.73 VS 35		12.98 B 12						
48 S 6	8.85 sh	9.00 VB 17		9.55 S 24	9.65 S 22	9.87 S 10	10.25 S 8			11.03 S 20	11.55 B 10	11.87 B 10	12.43 S 43	12.77 B 5	13.15 B 21				14.46 VB 10	
50 B 0	8.98 VB 31		9.37 S 22	9.57 S 10	9.73 VB 12		10.20 B 12	10.43 B 16	10.67 B 5	11.08 VB 20		11.78 S 31	12.08 B 10	12.50 B 10	12.83 VB 11	13.45 VB 22		14.28 VB 19		
46 B 7	8.94 VB 11		9.33 B 9		9.63 B 9	9.88 B 4	10.25 S 8	10.42 VB 3	10.67 B 6	11.00 B 3	11.16 S 8	11.58 B 23	12.34 B 22	12.84 VB 3	13.05 VB 7	13.60 S 20		14.28 VB 7		
.48 S 28	8.78 S 16	8.90 B 13	9.10 B 9	9.47 B 11		9.98 B 9	10.30 B 6		10.71 S 12		11.21 B 10	11.77 B 28	12.08 sh	12.45 S 20			13.77 B 16	14.20 VB 5		
.49 S 28	8.80 B 14			9.45 B 7	9.62 B 5	9.90 S 13		10.63 S 14		11.21 B 5	11.43 B 10	11.79 S 25	12.30 S 21	12.79 B 9			13.82 S 18			
.52 S 21	8.83 B 15	8.91 sh		9.43 B 10	9.53 sh	9.86 B 6		10.67 B 9		11.07 S 18	11.47 sh	11.75 S 32	12.05 VB 12	12.47 S 20	12.83 VB 3	13.15 B 7	13.45 VB 5	13.83 B 25	14.18 VB 1	14.37 VB 1
.53 S 20	8.80 S 14			9.45 B 15	9.67 VB 10	9.87 VB 7	10.15 B 3	10.54 S 15	10.87 S 14	11.23 B 5		11.75 S 31	12.33 S 27	12.75 S 3		13.45 B 4	13.86 S 24			15.08 VB 40
53 S 21	8.90 VB 11			9.45 VB 13	9.63 S 10			10.56 B 11	10.87 B 11			11.73 VB 31	12.34 B 17	12.75 B 3		13.45 B 4	13.87 S 24			
63 S '1	8.88 S 9	9.16 VS 34						10.64 S 13	10.96 S 18		11.38 S 18	11.90 S 7	12.61 S 10	12.90 B 12		13.70 sh	13.92 B 28		14.33 B 36	

that of any other peak in the same spectrum. The intensity variations, with increasing chain length, of absorption bands assigned to motions of specific functional groups may be followed, thus increasing the accuracy of the initial assignments. Inasmuch as the α-amino acids used in preparing TABLE 1 were extremely pure optically, the wave lengths of the tabulated absorption peaks may be used in deciding qualitatively the optical purity of a straight-chain α-amino acid or in establishing a quantitative analytical procedure for estimating the amount of an undesired isomer.

The first paper of this series[10] listed five absorption bands characteristic of the straight-chain L-amino acids and indicated that these absorption peaks reflected approximately the following motions of the functional groups:

2128 $cm.^{-1}$ (4.7 μ) — some motion of the charged α-amino group
1587 $cm.^{-1}$ (6.3 μ) — antisymmetric stretch ($—COO^-$)
1515 $cm.^{-1}$ (6.6 μ) — Amino Acid II band
1444 $cm.^{-1}$ (6.9 μ) — —CH_2 or —CH_3 deformation
1408 $cm.^{-1}$ (7.1 μ) — symmetric stretch (COO^-).

The data of TABLE 1 confirm these initial assignments, re-emphasize the pronounced influence of the polar form on the absorption spectrum, and, because of considerable improvement in over-all resolution, permit additional precise correlations between absorption peaks and the motions of specific functional groups.

Correlations between observed absorption peaks and the motions of known functional groups are complicated in the 3000 $cm.^{-1}$ region of the L-amino acids by the influence of hydrogen bridging, which shifts the frequencies at which the (N—H) stretching motions of the charged α-amino group absorb into the frequency region of the asymmetric and symmetric stretching motions of the methyl and methylene groups. It is, therefore, not possible with NaCl optics to resolve the stretching motions of the charged α-amino, the methyl, and the methylene groups. FIGURE 1 shows the absorption spectra of glycine and L-alanine; it is apparent that the glycine spectrum exhibits four adequately resolved peaks in the 3000 cm.$^{-1}$ region at 3096 cm.$^{-1}$ (3.23 μ), 2899 $cm.^{-1}$ (3.45 μ), 2801 $cm.^{-1}$ (3.57 μ), and 2564 $cm.^{-1}$ (3.90 μ). In the absence of a methyl group, the 3096 cm.$^{-1}$ peak must reflect predominantly the (N—H) stretch of the NH_3^+ group, whereas the 2899 cm.$^{-1}$ and 2802 cm.$^{-1}$ peaks may represent motions of the single methylene group. No specific assignment is made for the 2564 cm.$^{-1}$ band, but it is of interest to note that increasing the chain length diminishes the relative intensity of this peak. In contrast with glycine, the spectrum of L-alanine shows a 2941 cm.$^{-1}$ (3.40 μ) band, which is reduced in intensity and appears as a shoulder on the side of the sharp intense 3040 cm.$^{-1}$ (3.29 μ) peak. In the absence of a discrete methylene group in L-alanine, this observation is interpreted as indicating that absorption in the 2899 cm.$^{-1}$ (3.45 μ) region reflects predominantly motions of the methylene units of structure, and absorption in the 3077 cm.$^{-1}$ (3.25 μ) region, stretching motions of the charged α-amino group, with terminal methyl groups contributing to the long wave-length side of this absorption peak.[2] This assignment is supported by the observation (FIGURE 2) that, as the ratio of methylene to methyl and amino groups is increased by increasing the chain length in a ho-

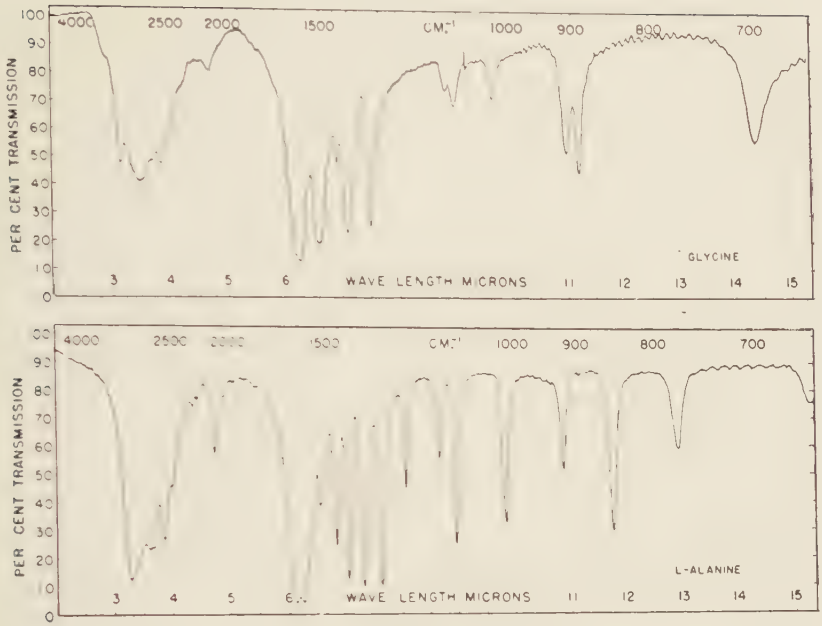


FIGURE 1

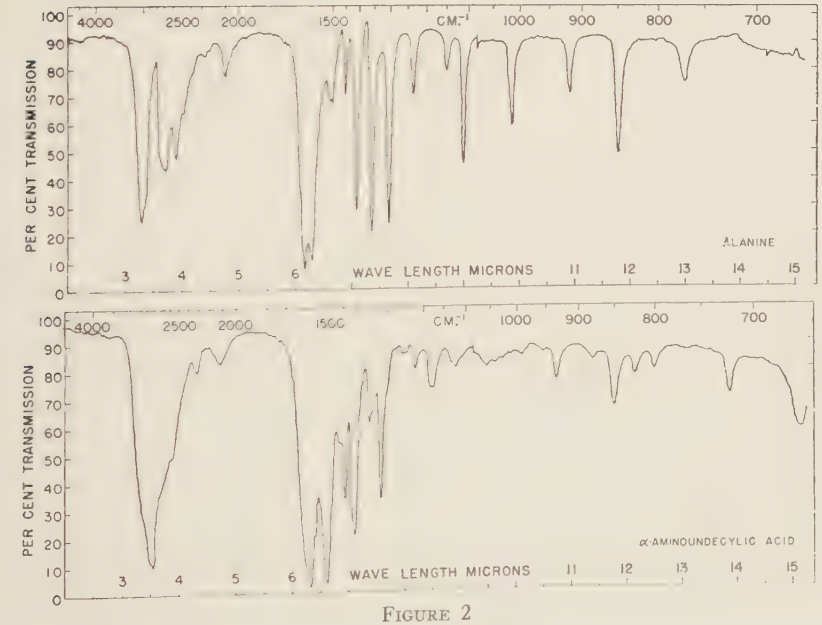


FIGURE 2

mologous fashion, the 3077 cm.$^{-1}$ band apparently diminishes in relative intensity, appearing finally as an inadequately resolved shoulder on the side of an intense band whose peak centers at approximately 2882 cm.$^{-1}$ (3.47 μ).[27] Inadequate resolution also makes it difficult to assign absorption frequencies to the symmetric stretching motions of these three functional groups. The 2801 cm.$^{-1}$ (3.57 μ) band of glycine most probably arises from the symmetric $-CH_2$ stretch; this peak is also influenced by increasing chain length and, in the spectra of the higher homologues, it usually appears as a shoulder on the side of the 2882 cm.$^{-1}$ (3.47 μ) band. It is important to note also that, except for glycine, each of the straight-chain optically active α-amino acids has a single (—C—H) group; although the low intensity arising from this motion is well recognized, it is suggested that the 2817 cm.$^{-1}$ (3.55 μ) band in L-alanine is a reflection of this unit or else of the symmetric stretching of the (CH_3—) group.[28, 29] Adequate resolution of the band due to the (—C—H) group seems to be possible only in small molecules.[2]

The 1650 to 667 cm.$^{-1}$ region. Between 1667 cm.$^{-1}$ (6.0 μ) and 667 cm.$^{-1}$ (15.0 μ) there are eleven adequately resolved absorption bands in the solid-state spectrum of glycine. Of these, three have been assigned to motions of functional groups responsible for the completely polar character of the α-amino acid; these are:

1600 cm.$^{-1}$ (6.25 μ) — antisymmetric stretch (COO^-)
1408 cm.$^{-1}$ (7.10 μ) — symmetric stretch (COO^-), and
1515 cm.$^{-1}$ (6.60 μ) — Amino Acid II band.[2]

In the absence of a methyl group, two of the remaining eight absorption bands are assigned to bending motions of the single methylene group: scissoring at 1443 cm.$^{-1}$ (6.93 μ) and wagging at 1330 cm.$^{-1}$ (7.52 μ). The data of TABLE 1 show that, with the single exception of L-alanine, all the amino acids absorb in the frequency range 1330 $\pm$ 9 cm.$^{-1}$, and that the intensity of this absorption, relative to that of the corresponding symmetric motion of the methyl group, at 1357 $\pm$ 2 cm.$^{-1}$ (7.37 μ), increases as the molecular weight of the amino acid increases. It is also clear that the intensity of the (CH_2) scissoring at 1445 $\pm$ 4 cm.$^{-1}$ varies in the same fashion with respect to the corresponding asymmetric motion of the methyl group at 1460 $\pm$ 4 cm.$^{-1}$ (6.85 μ). The frequency ranges of the asymmetric and symmetric deformation motions of the terminal methyl, and the scissoring and wagging vibrations of the methylene groups are defined, therefore, and the preciseness of the assignment is supported by the indicated intensity relationship.

The problem of making accurate assignments for the remaining absorption bands in the glycine spectrum is made difficult by (1) interaction within the unit cell of the crystal, which may cause band-splitting and (2) the fact that (C—C) and (C—N) stretching motions absorb in the same frequency regions. The 1130 cm.$^{-1}$ (8.85 μ) band is assigned to a rocking motion of the $-CH_2-$ group. The 1109 cm.$^{-1}$ (9.02 μ) peak may also be a reflection of some motion of the $-CH_2-$ group, or could be the (C—C) or (C—N) stretch. The intensity of absorption at 1111 $\pm$ 6 cm.$^{-1}$ (9.0 μ) seems to diminish with increasing chain length; this would suggest the involvement of the (C—N) bond. The 1033 cm.$^{-1}$ (9.68 μ) is assigned a ($-CH_2-$) motion (although again this seems low),

and the 913 cm.$^{-1}$ (10.98 μ) and 890 cm.$^{-1}$ (11.22 μ) bands to the (C—C) stretch. It is significant that in the spectrum of glycine there is no appreciable absorption between 890 cm.$^{-1}$ (11.22 μ) and 696 cm.$^{-1}$ (14.37 μ) and that with increasing chain length the number of sharp intense peaks within this frequency region increases sharply. In glycine the 696 cm.$^{-1}$ (14.37 μ) band is tentatively considered to be associated with some motion of the charged α-amino group.

L-Alanine, in contrast to glycine, is without a methylene group, but is characterized by a terminal methyl and a single C—H group. The differences in their spectra should reflect this difference in functional groups. The spectrum of L-alanine has fourteen discrete peaks between 1700 and 700 cm.$^{-1}$ (6.0 and 15.0 μ) and, as in glycine, the completely polar character is mirrored by absorption at the following frequencies:

1592 cm.$^{-1}$ (6.28 μ) — antisymmetric stretch (COO^-)
1412 cm.$^{-1}$ (7.08 μ) — symmetric stretch (COO^-)
1637 cm.$^{-1}$ (6.11 μ) and 1527 cm.$^{-1}$ (6.55 μ) — the Amino Acid I and II bands.[2]

The deformation motions of the terminal methyl group absorb at 1456 cm.$^{-1}$ (6.87 μ) and 1357 cm.$^{-1}$ (7.38 μ) for the asymmetric and symmetric motions, respectively. It is apparent that the deformation motions of the terminal methyl group of L-alanine absorb at significantly different frequencies than do the scissoring and wagging motions of the methylene group of glycine. The 1307 cm.$^{-1}$ (7.65 μ) peak of alanine seems to decrease in intensity with increasing chain length and, primarily for this reason, is assigned to a deformation motion of the lone C—H group. It is significant that the spectrum of glycine, in marked contrast to that of alanine, does not absorb at the following frequencies: 1456 cm.$^{-1}$ (6.87 μ), 1355 cm.$^{-1}$ (7.38 μ), and 1307 cm.$^{-1}$ (7.65 μ).

The following peaks — 1114 cm.$^{-1}$ (8.98 μ), 850 cm.$^{-1}$ (11.73 μ), 1016 cm.$^{-1}$ (9.83 μ), 1235 cm.$^{-1}$ (8.10 μ), and 769 cm.$^{-1}$ (12.98 μ) — arranged in order of decreasing relative intensity, are present in the spectrum of L-alanine and are considered to be produced by the following motions. The first peak, 1114 cm.$^{-1}$, is produced by an in-plane rocking motion of the methyl group; absorption in the region of 1125 ± 14 cm.$^{-1}$ is found in the spectra of all the α-amino acids from alanine to α-amino-*n*-dodecylic acid, and the intensity seems to decrease with increasing molecular weight. A similar observation may be made for absorption in the frequency regions 850 cm.$^{-1}$ and 770 cm.$^{-1}$. Absorption near 1017 cm.$^{-1}$ is observed in all the α-amino acids except α-amino-*n*-undecylic and α-amino-*n*-dodecylic. It is therefore considered that absorption in these frequency regions is associated with motions of the terminal methyl group or with motions of the terminal methyl group and the carbon-to-carbon skeletal chain. It is significant that alanine has absorption bands assigned to the terminal methyl group, corresponding to frequencies assigned to motions of the terminal methyl groups in the *n*-paraffins, and that in the latter compounds the methyl groups are not under the influence of a charged α-amino group or carboxylate ion.[30, 31]

In contrast to glycine and L-alanine, L-α-amino-*n*-butyric acid contains a terminal methyl and a β methylene group. Its solid-state spectrum should

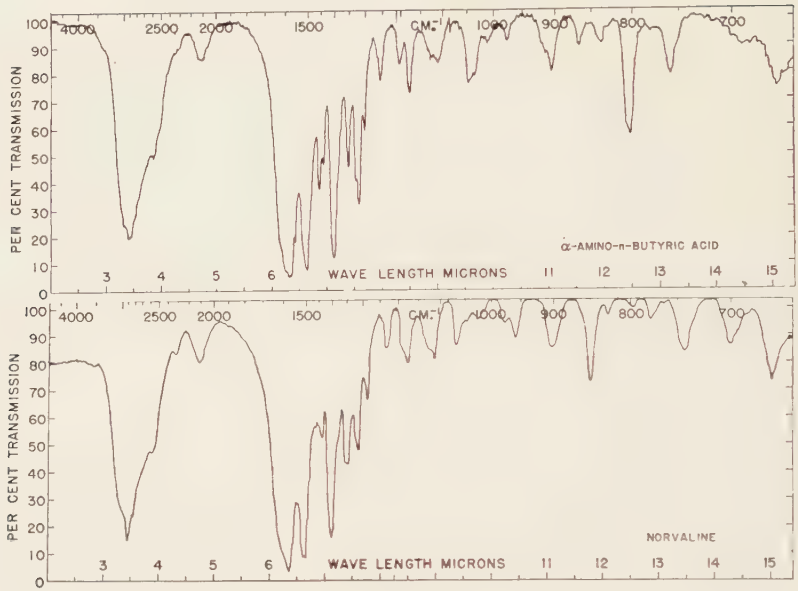


FIGURE 3

show absorption at frequencies that reflect this structural difference. The spectrum of α-amino-*n*-butyric acid (FIGURE 3) absorbs at the four frequencies that characterize the completely polar structure of the α-amino acids:

1588 cm.$^{-1}$ (6.33 μ) — antisymmetric stretch (COO^-)
1403 cm.$^{-1}$ (7.13 μ) — symmetric stretch (COO^-)
1605 cm.$^{-1}$ (6.23 μ) — Amino Acid I band
1506 cm.$^{-1}$ (6.64 μ) — Amino Acid II band.

The asymmetric (CH_3—) deformation absorbs at 1464 cm.$^{-1}$ (6.83 μ) and the observed intensity is greater than for the scissoring motion of the methylene group at 1445 cm.$^{-1}$ (6.92 μ). This is consistent with simple calculations, which show that the —CH_3 stretching mode has a greater net change in dipole moment than the (—CH_2—) stretching motion.[32] The corresponding symmetric bending vibrations of the methyl and methylene group absorb at 1355 cm.$^{-1}$ (7.38 μ) and 1321 cm.$^{-1}$ (7.57 μ). Adequately resolved absorption peaks at 1255 cm.$^{-1}$ (7.97 μ), 1111 cm.$^{-1}$ (9.00 μ), 1013 cm.$^{-1}$ (9.87 μ), 844 cm.$^{-1}$ (11.87 μ), and 784 cm.$^{-1}$ (12.77 μ) are assigned to motions of the terminal methyl group for the reasons discussed under alanine. It is significant to note that, in contrast to glycine and alanine, α-amino-*n*-butyric acid absorbs in the 769 cm.$^{-1}$ (13.0 μ) to 714 cm.$^{-1}$ (14.0 μ) range. The band at 763 cm.$^{-1}$ (13.15 μ) is assigned to some motion of the CH_3—CH_2— structure. In support of this assignment, the data of TABLE 1 indicate that, as the ratio of methylene to methyl groups in the structure is increased, the number of discrete absorption peaks in the 769 to 714 cm.$^{-1}$ region increases and the frequencies at which the bands absorb decrease. This type of absorption pattern in this region of the infrared is characteristic of the *n*-paraffins, and its appearance in the higher-molecu-

lar-weight straight-chain α-amino acids indicates the extent to which their polar character influences the characteristic $(-CH_2-)_n$ paraffinic absorption in the "fingerprint" region. Resolved absorption bands at 1209 cm.$^{-1}$ (8.27 μ), 1180 cm.$^{-1}$ (8.48 μ), 1047 cm.$^{-1}$ (9.55 μ), 975 cm.$^{-1}$ (10.25 μ), 865 cm.$^{-1}$ (11.55 μ), and 803 cm.$^{-1}$ (12.43 μ) identify the spectrum of L-α-amino-*n*-butyric acid and do not appear in the spectra of either glycine or L-alanine. Absorption at these frequencies is associated primarily with vibrations of the methylene groups. These assignments are in part supported by the compiled data of TABLE 1 which, on analysis, yield the following general observations characterizing the solid-state infrared absorption of this homologous series of straight-chain L-α-amino acids.

(1) The spectra of the α-amino acids from α-amino-*n*-butyric acid through α-amino-*n*-dodecylic acid appear to be characterized in the frequency region 1250 cm.$^{-1}$ to 1111 cm.$^{-1}$ (8.00 μ to 9.00 μ) by a triad of peaks near 1212 ± 25 cm.$^{-1}$, 1171 ± 11 cm.$^{-1}$, and 1126 ± 15 cm.$^{-1}$ As previously indicated, glycine absorbs strongly at 1130 cm.$^{-1}$ (8.85 μ) and 1109 cm.$^{-1}$ (9.02 μ), the latter peak being slightly more intense. In the absence of a methyl group, the 1109 cm.$^{-1}$ band is assigned to a rocking motion of the methylene group. The 1130 cm.$^{-1}$ peak may also be associated with $(-CH_2-)$ rocking or (C—C) stretch, or could possibly result from a splitting of peaks due to forces within the unit cell of the crystalline amino acid.[33] L-Alanine absorbs strongly in this region, at 1149 cm.$^{-1}$ (8.70 μ) and 1114 cm.$^{-1}$ (8.98 μ). The 1114 cm.$^{-1}$ peak is the more intense and is considered to arise from a rocking motion of the methyl group. In α-amino-*n*-butyric acid the typical three peaks are found close to 1209 cm.$^{-1}$ (8.27 μ), 1179 cm.$^{-1}$ (8.48 μ), and 1114 cm.$^{-1}$ (8.98 μ). The 1114 cm.$^{-1}$ band is assigned to a rocking motion of the terminal methyl group and the 1209 cm.$^{-1}$ and 1179 cm.$^{-1}$ bands to bending motions of the methylene group. The variation in the relative intensities within this triplet as the chain length is increased tends to confirm these assignments. With increasing chain length, absorption near 1120 cm.$^{-1}$ seems to decrease, while that in the region of 1171 cm.$^{-1}$ (8.54 μ) tends to increase and is usually stronger than the 1212 cm.$^{-1}$ (8.25 μ) peak.

(2) In the spectra of some of the higher members of this homologous series, specifically α-amino-*n*-octanoic, α-amino-*n*-decylic, α-amino-*n*-undecylic, and α-amino-*n*-dodecylic, there is a second characterizing triad of absorption peaks at 1282 cm.$^{-1}$ (7.80 μ), 1255 cm.$^{-1}$ (7.97 μ), and 1235 cm.$^{-1}$ (8.10 μ). The appearance of this approximately evenly spaced progression in the higher members of the homologous series suggests that they reflect twisting and wagging motions of the increasing number of methylene groups. A similar observation has been made for hydrocarbon chains.[34]

(3) All of the L-α-amino-*n*-acids having one or more methylene groups absorb in the frequency range 956 ± 20 cm.$^{-1}$ (10.45 μ). The frequency of the band decreases with increasing chain length, to 940 cm.$^{-1}$ in L-α-amino-*n*-dodecylic acid. Similarly, all the amino acids have an absorption peak near 803 ± 10 cm.$^{-1}$ (12.45 μ). Absorption in these two frequency regions is considered to reflect bending motions of the methylene groups.

(4) In making assignments for the absorption peaks found in the spectrum

of α-amino-*n*-butyric acid we have indicated that the frequency region between 769 cm.$^{-1}$ (13.0 μ) and 714 cm.$^{-1}$ (14.0 μ) is associated with vibrations of the structure (CH_2)—CH_3. The similarity of this absorption to that of the sequence CH_3 $(CH_2)_n$— in the straight-chain paraffins is striking. Specifically, when *n* in the paraffin series is 6, an absorption at 724 cm.$^{-1}$ (13.82 μ) to 720 cm.$^{-1}$ (13.86 μ) is observed; correspondingly, in L-α-amino-*n*-nonylic, —$(CH_2)_6$—, there is a broad band at 724 cm.$^{-1}$ (13.82 μ). When *n* in the paraffin series is 5, there is a characterizing band at 724 cm.$^{-1}$ (13.82 μ) to 720 cm.$^{-1}$ (13.84 μ), and in L-α-amino-*n*-octanoic there is a sharp peak at 724 cm.$^{-1}$ (13.82 μ); the correspondence of absorption in this region holds down the series to L-α-amino-*n*-butyric acid. In the paraffins, as *n* is made smaller, the frequency at which the CH_3 $(CH_2)_n$ structure absorbs increases. When $n = 1$, an absorption band is found at 769 cm.$^{-1}$ (13.00 μ). This is also true for the α-amino acids; L-α-amino-*n*-butyric has a strong broad absorption band at 743 cm.$^{-1}$ (13.45 μ).[30]

In L-α-amino-*n*-undecylic acid, the ratio of methylene to methyl groups is 8 to 1, and the influence that this preponderance of methylene units has on the characteristic absorption of a straight-chain α-amino acid will now be considered. It is apparent that the relative intensities of the three peaks reflecting the polar character — 1575 cm.$^{-1}$ (6.35 μ), antisymmetric stretch (COO^-); 1408 cm.$^{-1}$ (7.10 μ), symmetric stretch (COO^-); and 1515 cm.$^{-1}$ (6.61 μ), (C—N—H) — have diminished. However, their intensities relative to one another seem to have remained constant. The intensity at 1464 cm.$^{-1}$ (6.83 μ), asymmetric (CH_3—) deformation, is less than that at 1441 cm.$^{-1}$ (6.94 μ), scissoring (CH_2) motion. This same intensity relationship holds for the corresponding symmetric bending motions at 1357 cm.$^{-1}$ (7.37 μ), symmetric (CH_3—) deformation, and 1321 cm.$^{-1}$ (7.57 μ), wagging of (CH_2). In contrast to the spectrum of glycine, in which there is no resolved absorption from 1330 cm.$^{-1}$ (7.52 μ), wagging of (—CH_2—), to 1130 cm.$^{-1}$ (8.85 μ), (—CH_2—) rocking, there are six adequately resolved bands at 1269 cm.$^{-1}$ (7.88 μ), 1255 cm.$^{-1}$ (7.97 μ), 1230 cm.$^{-1}$ (8.13 μ), 1188 cm.$^{-1}$ (8.42 μ), 1172 cm.$^{-1}$ (8.53 μ), and 1124 cm.$^{-1}$ (8.90 μ). Absorption at these frequencies must reflect motions of either the terminal methyl or the (CH_2) groups, and specific assignments follow from those previously indicated for the lower members of this series of homologous L-α-amino-*n*-acids: absorption at 1188 cm.$^{-1}$ and 1172 cm.$^{-1}$ to wagging or twisting motions of the (CH_2) groups, and at 1230 cm.$^{-1}$ and 1124 cm.$^{-1}$ to deformation motions of the terminal methyl group. Absorption at 1269 cm.$^{-1}$ and 1115 cm.$^{-1}$ is attributed to the influence of $(—CH_2—)_8$, since these bands are prominent in the spectrum of L-α-amino-*n*-butyric acid. In this respect the data of TABLE 1 indicate that, beginning with L-α-amino-*n*-octanoic acid, there is a characteristic series of weakly absorbing but adequately resolved peaks at 1284 cm.$^{-1}$ (7.79 μ), 1253 cm.$^{-1}$ (7.98 μ), 1239 cm.$^{-1}$ (8.07 μ), 1205 cm.$^{-1}$ (8.30 μ), 1179 cm.$^{-1}$ (8.49 μ), and 1136 cm.$^{-1}$ (8.80 μ).

Absorption in the frequency range 714 cm.$^{-1}$ (14.0 μ) to 667 cm.$^{-1}$ (15.0 μ) requires additional comment.[35, 36] It has been shown that whenever a CH_3—CH_2— unit of structure is present there will be one or more intense, adequately

resolved bands in the 769 cm.$^{-1}$ (13.0 μ) to 714 cm.$^{-1}$ (14.0 μ) region and that as the number of (—CH_2—) units is increased the number of peaks in this region also increases. Comparison of the spectra of L-α-amino-*n*-butyric acid and L-norleucine with those of L-α-amino-*n*-nonylic acid and L-α-amino-*n*-undecylic acid in this region (TABLE 1) suggests that in the straight-chain L-α-amino acids the contribution of the CH_3—$(CH_2)_n$— structure to absorption below 714 cm.$^{-1}$ is small.[35, 36]

Glycine, L-α-amino-*n*-butyric acid, L-norvaline, L-norleucine, L-α-amino-*n*-nonylic acid, and L-α-amino-*n*-dodecylic acid all have an absorption peak in the frequency range 696 ± 4 cm.$^{-1}$ (14.37 μ). It is suggested, therefore, that absorption in this frequency range reflects some motion of the charged α-amino group.

TABLE 1 and FIGURES 1 through 4 contain data that also permit precise identification of the absorption peaks produced by the symmetric bending motions of the hydrogens of the methyl and methylene groups. The spectrum of glycine, which contains a single methylene group, has a sharp intense band at 1330 cm.$^{-1}$ (7.52 μ) and no other strongly absorbing band until 1170 cm.$^{-1}$ (8.55 μ) is reached. L-Alanine, which contains a single methyl unit and a single —C—H unit of structure, has two very sharp peaks of almost the same intensity, 1355 cm.$^{-1}$ (7.38 μ) and 1307 cm.$^{-1}$ (7.65 μ). L-α-Aminobutyric acid, in which the ratio of methyl units of structure to methylene units is 1, absorbs at 1355 cm.$^{-1}$ and 1321 cm.$^{-1}$ (7.57 μ), the latter peak having the greater intensity; in norvaline, where the ratio is 2, absorption peaks appear at 1355 cm.$^{-1}$ and 1319 cm.$^{-1}$, with the latter peak slightly more intense than the former. Norleucine, with a ratio of 3, absorbs at 1355 cm.$^{-1}$ and 1321 cm.$^{-1}$; both peaks are sharp, with the peak of lower frequency having a much greater intensity. Except for L-α-aminododecylic acid, the remaining members of this homologous series exhibit absorption peaks at 1357 cm.$^{-1}$ and 1321 cm.$^{-1}$, with the intensity of the latter peak being the greater. As far as intensity is concerned, it is apparent that, as in the case of the asymmetric motions, integration of the areas within the specific peaks 1357 cm.$^{-1}$ and 1321 cm.$^{-1}$ would suggest the number of methylene and methyl units of structure in the molecule, and also indicate that the absolute intensity of the terminal methyl group is greater than that of the methylene unit. These observations require that the 1357 cm.$^{-1}$ peak be assigned to the symmetric deformation motion of the hydrogens of the terminal methyl group, and the 1321 cm.$^{-1}$ peak to wagging of the methylene. Correlating the 1357 cm.$^{-1}$ peak specifically with the symmetric deformation of the terminal methyl group emphasizes that the terminal methyl moiety in the straight-chained α-amino acids, absorbs at lower frequencies than the terminal methyl groups of *n*-paraffin hydrocarbons and that its intensity is not independent of chain length.[37] These differences demonstrate the influence of the zwitterion structure of the α-amino acid on the intensity and frequency of the symmetric and asymmetric motions of its terminal methyl group and on the bending vibrations of its methylene groups. The nature of this influence is not clear, but the charged α-amino group and the carboxylate ion with its double-bond character must alter the force fields about the terminal methyl group and the methylene units. As we have previously indicated, the bending mo-

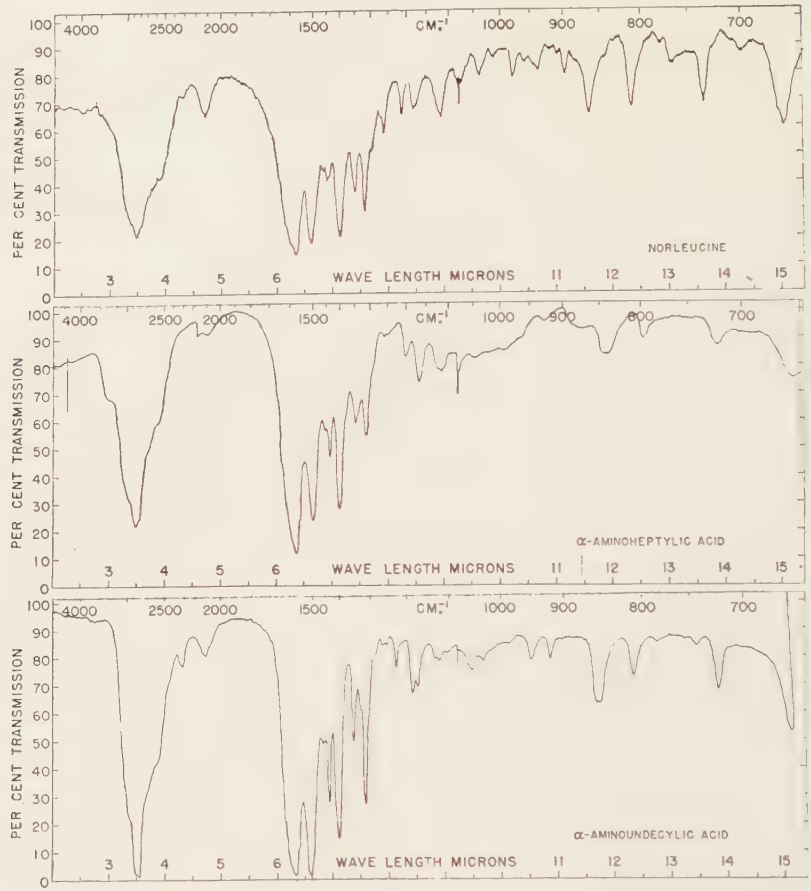


FIGURE 4

tions of the (CH_3) group and the (CH_2) groups in L-α-amino-*n*-dodecylic acid appear to absorb atypically when compared to the lower members of this homologous series. In L-α-aminododecylic acid, the ratio of methylene units to methyl units is 9, but its spectrum shows no 1357 cm.$^{-1}$ (7.37 μ) or 1321 cm.$^{-1}$ (7.57 μ) peak; instead, there is the usual strong band at 1464 cm.$^{-1}$ (6.83 μ) and two sharp intense peaks at 1346 cm.$^{-1}$ (7.43 μ) and 1338 cm.$^{-1}$ (7.47 μ). In view of the characteristic absorption of L-α-aminoundecylic acid, this apparent anomaly is difficult to understand, but it most probably reflects the diminishing influence of the dipolar ion structure and the preponderance of the methylene groups.

Using glycine and L-alanine as models and drawing upon the absorption spectra of the remaining ten straight-chained L-α-amino acids examined, it is apparent that it is possible to assign adequately resolved absorption peaks to motions of specific functional groups known to be present; the accuracy of

these assignments is precise in the 1700 to 1300 cm.$^{-1}$ region, less precise in the 4000 to 2000 cm.$^{-1}$ region, and tentative in the "fingerprint" region.

Characterization of the Solid-State Absorption of the Racemic Form of the Straight-Chain α-Amino Acids

It is well recognized that the solid-state spectra of the racemic and optically active forms of the straight-chain α-amino acids differ.[38,39] Correlations are lacking, however, between the observed differences in spectral absorption peaks and vibrations known to occur in the optically active isomers. TABLE 2 lists all the absorption bands observed in the solid-state spectra of six racemic straight-chain α-amino acids, and a comparison of these data with those of TABLE 1 gives some indication of the extent to which optical isomerism influences the solid-state spectra. The spectrum of the racemic form of alanine differs very little from that of its optically active form (FIGURE 5). The spectrum of the racemic form shows an adequately resolved peak at 1028 cm.$^{-1}$ (9.73 μ) which is absent in the active form and, in the 1667 cm.$^{-1}$ (6.0 μ) region, the Amino Acid I band at 1647 cm.$^{-1}$ (6.07 μ) appears more intense and more adequately resolved from the 1595 cm.$^{-1}$ (6.27 μ) assigned to the antisymmetric (COO^-) peak. In all other frequency regions the spectra appear to be identical. The spectra of the racemic and active forms of α-amino-*n*-butyric acid (FIGURE 6) differ more significantly than the corresponding spectra of alanine. The spectrum of the racemic compound, in contrast to that of the L-form, is characterized by an intense, adequately resolved peak at 1661 cm.$^{-1}$ (6.02 μ), for which no assignment is made at present. The intensity of the 1515 cm.$^{-1}$ (6.60 μ) Amino Acid II band, is much less than that of the adjacent 1587 cm.$^{-1}$ (6.30 μ) peak, the antisymmetric (COO^-) stretch. In the region below 1400 cm.$^{-1}$ there is a sharp peak at 1379 cm.$^{-1}$ (7.25 μ), an intense sharp peak at 1339 cm.$^{-1}$ (7.47 μ), an adequately resolved shoulder on the high-frequency side of this peak at 1355 cm.$^{-1}$ (7.38 μ), a very intense sharp peak at 1321 cm.$^{-1}$ (7.57 μ) (methylene wagging), and a weakly absorbing peak at 1272 cm.$^{-1}$ (7.86 μ). In the spectral region 1379 cm.$^{-1}$ to 1321 cm.$^{-1}$ the enantiomorph absorbs only at 1357 cm.$^{-1}$ (7.37 μ), the symmetric ($—CH_3$) deformation, and at 1322 cm.$^{-1}$ (7.57 μ), the ($-CH_2-$) wagging. In the 1667 cm.$^{-1}$ (6.0 μ) region there is no absorption at 1661 cm.$^{-1}$ (6.02 μ), and the intensity of the Amino Acid II band at 1515 cm.$^{-1}$ (6.60 μ) is close to that of the antisymmetric (COO^-) band at 1580 cm.$^{-1}$ (6.33 μ). These observations indicate clearly both the extent to which optical isomerism influences solid-state spectra and which of the vibrations are especially perturbed. The spectrum of the racemic form of α-amino-*n*-butyric acid is further characterized by the intensity of the 1159 cm.$^{-1}$ (8.63 μ) band, which is very close to that of the 1136 cm.$^{-1}$ (8.83 μ), methyl rocking, and by the absence of absorption at 1034 cm.$^{-1}$ (9.67 μ).

In the spectrum of the racemic form of norvaline (FIGURE 7), as in that of the racemic form of α-amino-*n*-butyric acid, there is a resolved absorption peak at 1661 cm.$^{-1}$ (6.02 μ) and the intensity of the Amino Acid II band at 1522 cm.$^{-1}$ (6.57 μ) is much less than that of the antisymmetric (COO^-) at 1582 cm.$^{-1}$ (6.32 μ). In the racemic compound the intensity of the 1475 cm.$^{-1}$ (6.78 μ)

TAB

ABSORPTION OF THE RACEMIC

DL-Alanine																														
λ			3.25	3.55			3.73	3.85	4.75			6.13	6.28			6.55			6.88	7.07			7.36				7.63			
Desc.			VB	B			B	S	S			S	S			S			VS	VS			VS				VS			
% A			40	33			30	25	18			29	62			20			38	47			51				49			
DL-α-Amino-*n*-butyric acid																														
λ				3.40			3.77	3.90	4.75	6.01	6.12	6.29				6.58	6.78	6.89	7.03	7.24	7.37	7.47	7.56							
Desc.				VB			sh	sh	B	S	sh	S				S	S	S	VS	S	sh	S	VS							
% A				41					10	37		51				30	23	20	39	19		35	40							
DL-Norvaline																														
λ	3.35			3.42	3.65	3.77	3.89	4.75	6.02	6.17				6.30	6.57	6.78	6.86	7.04			7.38	7.44				7.63	7.73			
Desc.	sh			VB	sh	sh	sh	B	S	sh				S	S	S	S	VS			S	S				sh	VS			
% A				75				22	67					82	55	40	29	75			58	66					45			
DL-Norleucine																														
λ				3.44			3.75	3.87	4.75	6.00	6.16			6.30	6.57	6.78	6.85	7.04			7.35	7.45				7.67	7.73			
Desc.				VB			sh	sh	B	S	sh			S	S	S	S	VS			S	S				sh	VS			
% A				54					12	50				67	30	25	22	62			46	53					30			
DL-α-Amino-*n*-heptylic acid																														
λ				3.43	3.51	3.75	3.87	4.75	6.02			6.20	6.30	6.57	6.78			7.04			7.40	7.52	7.61				7.84			
Desc.				VB	sh	sh	sh	B	S			sh	S	S	S			VS			VS	S	S				S			
% A				55				17	38				60	38	25			51			48	39	30				25			
DL-α-Amino-*n*-octanoic acid																														
λ				3.44	3.52	3.75	3.86	4.75	6.00	6.15	6.28			6.57	6.77	6.82		7.02	7.35	7.43			7.68				7.91			
Desc.				VB	sh	sh	sh	B	S	sh	S			S	S	sh		VS	S	S			S				VS			
% A				65				16	59		74			50	39			65	58	62			41				22			

% A = Per cent absorption; B = broad; S = strong; sh = shoulder; VB = very broad; VS = very strong.

band, asymmetric ($-CH_3$) deformation appears to be greater than that of the 1449 cm.$^{-1}$ (6.90 μ) peak, scissoring (CH_2); and the intensity of the 1359 cm.$^{-1}$ (7.36 μ) band, symmetric ($-CH_3$) deformation, is less than that of the 1342 cm.$^{-1}$ (7.45 μ) band, ($-CH_2-$) wagging. This relation of relative intensities is opposite to that seen in the spectrum of the corresponding L-form. The spectrum of the racemic compound is further characterized by a sharp intense band at 1159 cm.$^{-1}$ (8.63 μ) and, beginning with this peak, is very dissimilar to that of the L-form from 1250 to 667 cm.$^{-1}$ (8.0 to 15 μ).

A comparison of the solid-state spectra of the racemic compound and L-form of norleucine (FIGURE 8) re-emphasizes the observations that, in the 4000 to 1650 cm.$^{-1}$ region, differences between the racemic and optically active straight-chain α-amino acids are slight, and that the spectrum of the racemic mixture seems to be characterized by (1) a sharp, intense, adequately resolved band at 1658 cm.$^{-1}$ (6.03 μ); (2) a striking difference in the intensity of the Amino Acid II peak relative to the antisymmetric (COO^-) band at 1580 cm.$^{-1}$ (6.33 μ); and (3) distinctive differences at those spectral frequencies known to correspond to the bending motions of the methyl and methylene groups. In the spectrum of racemic norleucine the intensity of the 1473 cm.$^{-1}$ (6.79 μ) peak,

LE 2

STRAIGHT-CHAIN α-AMINO ACIDS

8.07 S 22		8.67 S 20	8.96 VS 41			9.71 S 15	9.83 S 25			10.87 S 14			11.74 S 38			12.98 S 29					
7.85 S 15	8.32 S 12	8.64 S 16	8.86 S 17		9.46 S 14	9.78 S 8		10.17 S 6		10.87 B 10		11.49 B 6	11.83 B 5	12.38 S 22	12.80 B 5	13.06 B 9	13.24 sh			14.46 B 9	
8.04 S 32	8.34 S 23	8.64 S 32	8.93 S 32	9.26 VS 46					10.41 S 66	10.80 B 30		11.52 B 30		12.55 S 13		12.95 S 14		13.78 B 28	14.31 VB 36		
8.05 VS 17	8.36 S 11	8.62 S 19	8.90 S 18	9.28 VS 32					10.41 S 9	10.80 S 18		11.49 S 20		12.55 S 7	12.95 S 9			13.77 S 20	14.34 S 31		
8.12 S 21	8.40 S 12	8.61 S 19	8.88 S 23	9.25 S 25	9.44 B 6		9.98 B 9	10.23 B 4	10.67 S 10		11.14 S 17	11.34 S 10		12.01 S 5	12.86 S 8			13.85 S 14	14.30 B 22		14.97 S 5
8.10 VS 21	8.43 S 11	8.66 S 28	8.87 VS 19	9.21 VS 49		9.74 S 4	10.07 B 5		10.39 S 17	10.95 sh	11.06 S 27	11.23 sh	11.60 S 17	12.54 S 16	12.91 S 14			13.85 S 30	14.28 S 45		14.96 S 14

assigned to the asymmetric (—CH_3) deformation, appears to be equal to or slightly greater than the 1456 cm.$^{-1}$ (6.87 μ) peak assigned to a scissoring motion of the methylene groups. This observation again stresses the fact that in the spectra of the racemic forms it does not seem possible to establish a relation between the relative intensities of the peaks associated with the bending vibrations of the methyl or methylene groups and the chain length. The following absorption peaks serve specifically to differentiate the spectrum of racemic norleucine from that of the L-form, namely, 1160 cm.$^{-1}$ (8.62 μ), 920 cm.$^{-1}$ (10.85 μ), and 800 cm.$^{-1}$ (12.53 μ). The spectrum of the L-form of norleucine absorbs at 1127 cm.$^{-1}$ (8.87 μ), assigned to a rocking motion of the terminal methyl, and it is suggested that the characterizing band of the racemic form at 1159 cm.$^{-1}$ (8.63 μ) reflects the same motion. If this assignment is correct, it is further suggested that this peak arises from the splitting of a degeneracy present in the unit cell of the crystalline L-form.

The solid-state spectra of the racemic form of α-amino-*n*-heptylic and α-amino-*n*-octanoic acids (FIGURES 9 and 10) exhibit the absorption pattern that is characteristic of this optical form of the straight-chain α-amino acids. In specific contrast to its L-form, the spectrum of racemic α-amino-*n*-heptylic acid is further identifiable by absorption peaks at 699 cm.$^{-1}$ (14.30 μ), 781 cm.$^{-1}$

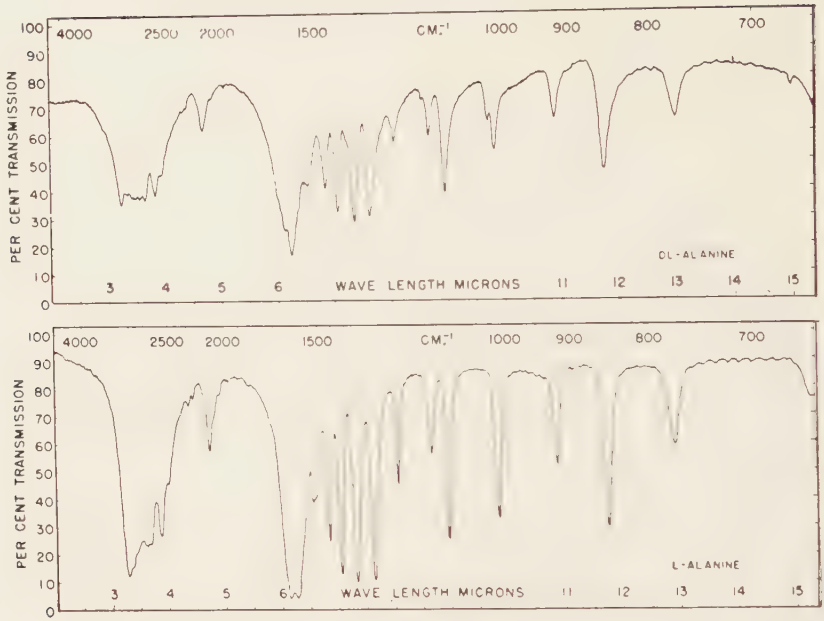


FIGURE 5

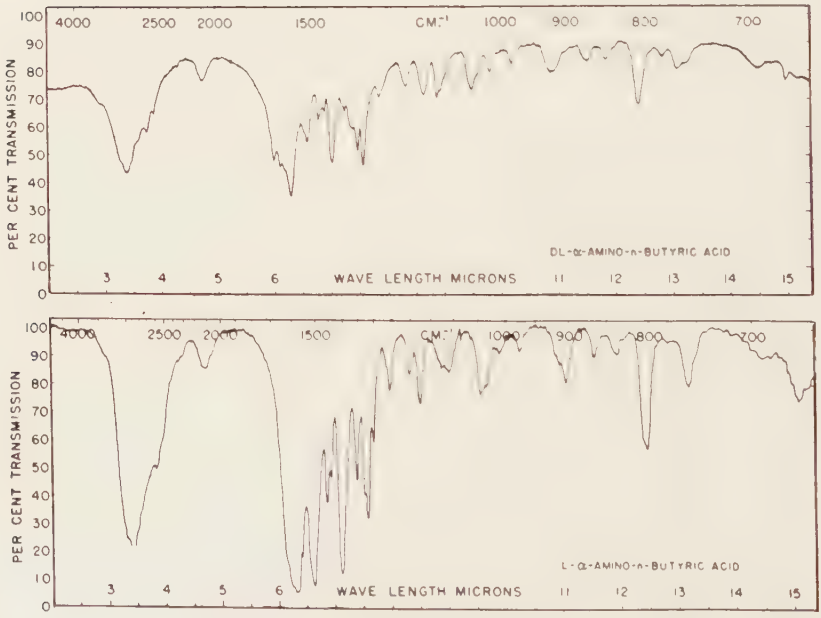


FIGURE 6

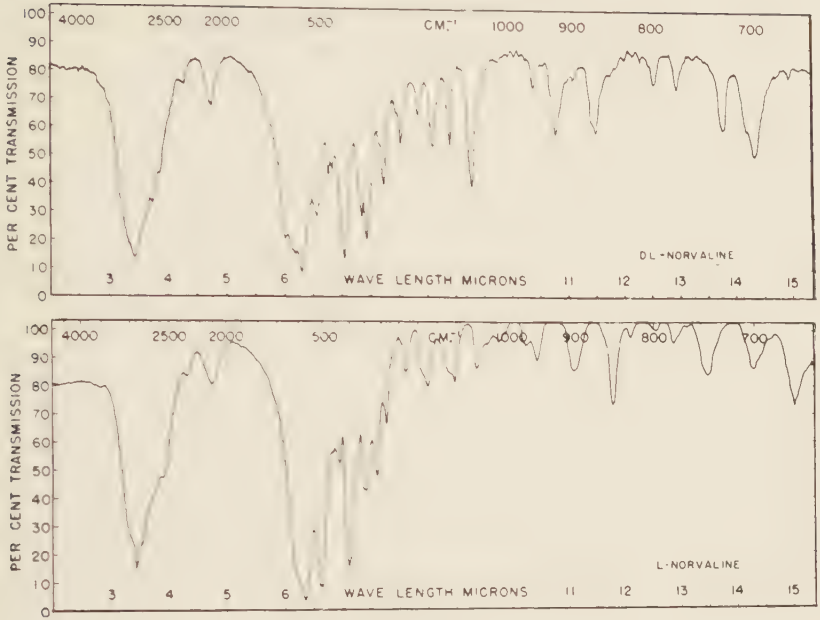


FIGURE 7

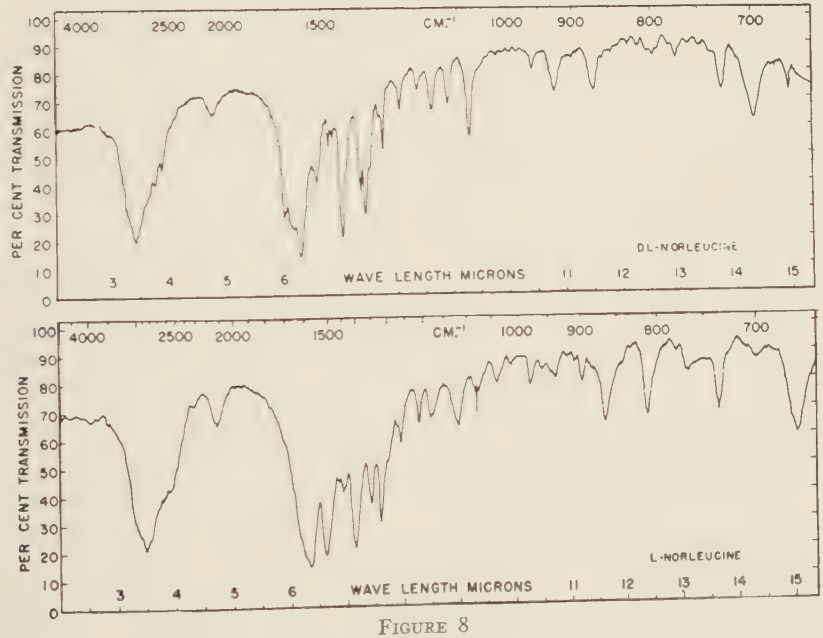


FIGURE 8

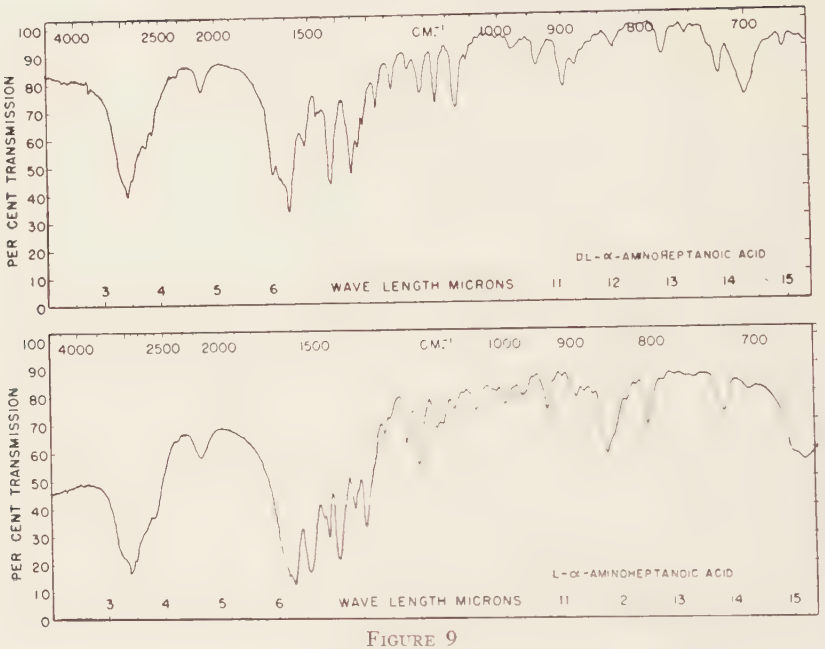


FIGURE 9

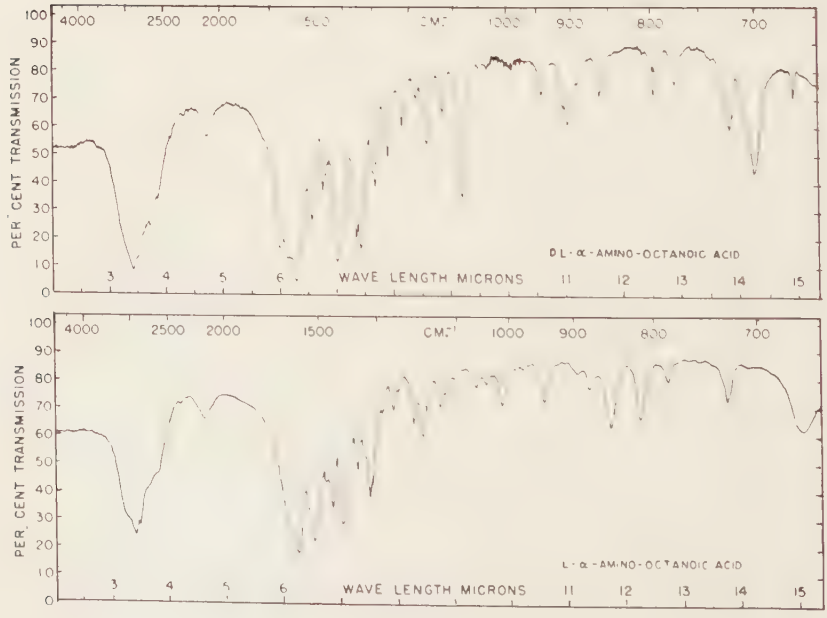


FIGURE 10

(12.85 μ), 833 cm.$^{-1}$ (12.00 μ), 901 cm.$^{-1}$ (11.10 μ), 1082 cm.$^{-1}$ (9.24 μ), and by the further observation that the intensity of the 1353 cm.$^{-1}$ (7.39 μ) peak is greater than that of the 1332 cm.$^{-1}$ (7.51 μ) peak. In the same way the spectrum of DL-α-amino-*n*-octanoic acid is identified by absorption peaks at the following frequencies: 700 cm.$^{-1}$ (14.27 μ), 775 cm.$^{-1}$ (12.90 μ), 800 cm.$^{-1}$ (12.53 μ), 999 cm.$^{-1}$ (11.00 μ), and 1082 cm.$^{-1}$ (9.23 μ).

With increasing chain length, starting with DL-norleucine, the spectra of the DL straight-chain α-amino acids are distinguished in the frequency region 1111 cm.$^{-1}$ (9.0 μ) to 667 cm.$^{-1}$ (15.0 μ) by an increase in the intensity of the absorption near 1075 cm.$^{-1}$ (9.30 μ) and 700 cm.$^{-1}$ (14.30 μ). Corresponding peaks in the L-forms tend to decrease in intensity until in the spectrum of L-α-amino-*n*-octanoic acid there is little or no absorption at these frequencies.

These characterizing differences between the solid-state spectra of racemic and optically active forms serve to emphasize the necessity for comparing only the spectra of structures that are both optically pure and optically alike; and it seems clear that failure to recognize this restriction is in part responsible for some of the current ambiguity in group-frequency assignments. The frequency assignment and relative intensities of the Amino Acid I and II bands are a case in point, for it is generally agreed that all α-amino acids in the polar form exhibit two characteristic absorption peaks in the frequency range 1600 cm.$^{-1}$ (6.02 μ) to 1500 cm.$^{-1}$ (6.67 μ), in addition to the antisymmetric (COO^-) band at 1580 cm.$^{-1}$ (6.33 μ).[40-42] The first of these peaks, at 1640 cm.$^{-1}$ (6.06 μ) to 1610 cm.$^{-1}$ (6.21 μ), the Amino Acid I band, is usually of low intensity or appears as a shoulder on the high-frequency side of the antisymmetric (COO^-) band at 1580 cm.$^{-1}$ (6.33 μ). The second of these peaks, the Amino Acid II band, absorbs near 1550 cm.$^{-1}$ (6.45 μ) to 1485 cm.$^{-1}$ (6.74 μ) and is usually more intense. There is considerably more agreement on the occurrence and relative intensity of the latter band than on the Amino Acid I band. Some authors have observed this peak in all the α-amino acids examined by them,[40-42] while others have failed to comment on it in relation to their spectra[43] or else have observed it with only a few compounds.[44] It has been suggested that these observational differences arise either from the inherently low intensity of the Amino Acid I band or are associated with differences in the optical forms examined.[2] The summarized data of TABLES 1 and 2 of this paper indicate that the latter explanation is correct. In the spectrum of the optically active form, the Amino Acid I band appears as an adequately resolved shoulder on the high-frequency side of the antisymmetric (COO^-) peak at 1587 cm.$^{-1}$ (6.30 μ), and the Amino Acid II band, the intensity of which approximates the antisymmetric (COO^-) peak, absorbs at 1515 cm.$^{-1}$. In contrast, it is seen that in the spectrum of the racemic compound, the Amino Acid II band still absorbs near 1515 cm.$^{-1}$ (6.60 μ), but its intensity relative to the antisymmetric (COO^-) peak has undergone a striking decrease. The Amino Acid I band appears to be still present as a shoulder on the high-frequency side of the antisymmetric (COO^-) band; however, a new, sharp, adequately resolved peak is now observed near 1658 cm.$^{-1}$ (6.03 μ). It is suggested that the appearance of this new peak explains the extended range given by Bellamy[2] for the Amino Acid I band. It is significant to

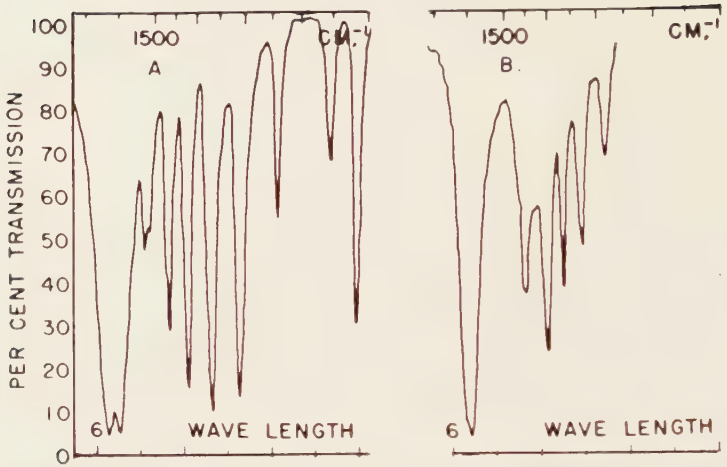


FIGURE 11. Absorption of optically active alanine in the solid state (A) and optically active alanine in D_2O solution (B). It is interesting to note that the frequencies of the anti symmetric and symmetric stretching motions of the carboxylate ion are not significantly different.

note that the frequency of the peak intensity of the antisymmetric (COO^-) band is essentially the same in both the optically active and racemic forms. The extent to which these stretching motions of the carboxyl ion are perturbed by the solid state is indicated in FIGURE 11, in which the absorption of optically active alanine in the solid state is compared with its approximately 0.01 *M* D_2O solution. The frequency of the symmetric stretching motion is not significantly altered and that of the antisymmetric motion is increased by only 25 cm.$^{-1}$, indicating that the influence of forces within the unit cell upon these vibrations is small. This comparison of curves A and B (FIGURE 11) also lends support to the assignment made for the Amino Acid I and II bands. These absorption bands are absent in the spectrum of D_2O solution (curve B), suggesting that the frequency of the motions that they reflect shifts on deuteration.

It is informative to consider these observed differences in the solid-state absorption of the optically active and racemic α-amino acids in terms of solid-state X-ray diffraction data. X-ray studies, refined by three-dimensional Fourier methods, of some of the simple α-amino acids substantiate the completely polar character of these molecules and indicate that in the crystalline state they are usually hydrogen-bonded by three strong intermolecular nitrogen-to-oxygen bridges.[15,16] These three bonds are disposed tetrahedrally about the $-NH_3^+$ group, with the atoms of the carboxyl group engaged unequally by different $-NH_3^+$ groups. A logical extension of these X-ray data suggests that, if differences in the solid-state absorption of the optically active and racemic α-amino acids are observed, they should occur in those frequency regions that correspond to hydrogenic bending vibrations of the charged $-NH_3^+$ group. The data of TABLES 1 and 2 support this inference and indicate that additional solid-state infrared absorption studies on homologous series of

chemically and optically pure model structures will both increase the precision of group-frequency assignments and enhance the usefulness of this technique in its application to problems of molecular structure.

References

1. Sutherland, G. B. B. M. 1952. Advances in Protein Chem. **7:** 291.
2. Bellamy, L. J. 1954. The Infrared Spectra of Complex Molecules. Wiley & Sons. New York, N. Y.
3. Jones, R. N. & C. Sandorfy. 1956. Technique of Organic Chemistry. Vol. 9. Chemical Applications of Spectroscopy : 247. Interscience. New York, N. Y.
4. Springall, A. D. 1954. The Structural Chemistry of Proteins. Chap. III. Academic Press. New York, N. Y.
5. Gore, R. C. & *in part* E. S. Waight. 1955. Determination of Organic Structures by Physical Means. Chap. V. Academic Press. New York, N. Y.
6. Sutherland, G. B. B. M. 1950. Discussions Faraday Soc **9:** 274.
7. Fraser, R. D. B. & W. C. Price. 1952. Nature. **270:** 490.
8. Wright, N. 1937. J. Biol. Chem. **120:** 641.
9. Sutherland, G. B. B. M. 1950. Discussions Faraday Soc. **9:** 319.
10. Koegel, R. J., J. P. Greenstein, M. Winitz, S. M. Birnbaum & R. A. McCallum. 1955. J. Am. Chem. Soc. **77:** 5708.
11. Ling, C. Y., S. Krimm & G. B. B. M. Sutherland. 1957. J. Chem. Phys. **25:** 543.
12. Stimson, M. M. & M. J. O'Donnell. 1952. J. Am. Chem. Soc. **74:** 1805.
13. Schiedt, U. & H. Reinwein. 1952. Z. Naturforsch. **7b:** 270.
14. Gross, P. C., J. Burnham & P. A. Leighton. 1937. J. Am. Chem. Soc. **59:** 1134.
15. Greenstein, J. P. 1954. Advances in Protein Chem. **9:** 121.
16. Otey, M. C., J. P. Greenstein, M. Winitz & S. M. Birnbaum. 1955. J. Am. Chem. Soc. **77:** 2112.
17. Greenstein, J. P., S. M. Birnbaum & M. C. Otey. 1953. J. Biol. Chem. **204:** 307.
18. Edsall, J. T. 1936. J. Chem. Phys. **4:** 1.
19. Edsall, J. T. 1937a. J. Phys. Chem. **41:** 133.
20. Edsall, J. T. 1937b. J. Chem. Phys. **5:** 225, 508.
21. Edsall, J. T. 1938. Cold Spring Harbor Symposia Quant. Biol. **6:** 40.
22. Edsall, J. T. & H. Scheinberg. 1940. J. Chem. Phys. **8:** 520.
23. Edsall, J. T. 1943. J. Am Chem. Soc. **65:** 1767.
24. Edsall, J. T., J. W. Otvos & A. Rich. 1950. J. Am. Chem. Soc. **71:** 474.
25. Gore, R. C., R. B. Barnes & E. Petersen. 1949. Anal. Chem **21:** 382.
26. Lacher, J. R., V. C. Croy, A. Kianpour & J. D. Park. 1954. J. Phys. Chem. **58:** 206.
27. Fuson, N., M. L. Josien & R. L. Powell. 1952. J. Am. Chem. Soc. **74:** 1.
28. Fox, J. J. & A. E. Martin. 1938. Proc. Roy. Soc. London. **A167:** 257.
29. Fox, J. J. & A. E. Martin. 1940. Proc. Roy. Soc. London. **A175:** 208.
30. McMurray, H. L. & V. Thornton. 1952. Anal. Chem. **24:** 318.
31. Sheppard, N. & D. M. Simpson. 1953. Quart. Revs. London **7:** 19.
32. Pozefsky, A. & N. D. Coggeshall. 1951. Anal. Chem. **23:** 1611.
33. Liang, C. Y., S. Krimm & G. B. B. M. Sutherland. 1956. J. Chem. Phys. **25:** 543.
34. Jones, R. N., A. F. McKay & R. G. Sinclair. 1952. J. Am. Chem. Soc **74:** 2575.
35. Sheppard, N. & G. B. B. M. Sutherland. 1947. Nature. **159:** 739.
36. Blout, E. R. & S. G. Linsley. 1952. J. Am. Chem. Soc. **74:** 1946.
37. Jones, R. N. 1955. Record Chem. Progr. Kresge-Hooker Sci. Lib. **16:** 278.
38. Wright, N. 1937. J. Biol. Chem. **120:** 641.
39. Wright, N. 1939. J. Biol. Chem. **127:** 137.
40. Randall, H. M., R. G. Fowler, N. Fuson & R. Dangl. 1949. The Infrared Determination of Organic Structures. Van Nostrand. New York, N. Y.
41. Lenormant, H. 1946. J. chim. phys. **43:** 327.
42. Fuson, N., M. L. Josien & R. L. Powell. 1952. J. Am. Chem. Soc. **74:** 1.
43. Klotz, I. M. & D. M. Gruen. 1948. J. Phys. & Colloid Chem. **53:** 961.
44. Thompson, H. W., D. L. Nicholson & N. L. Short. 1950. Discussions Faraday Soc. **9:** 222.
45. Levy, H. A. & R. B. Corey. 1941. J. Am. Chem. Soc. **63:** 2095.
46. Donohue, J. 1950. J. Am. Chem. Soc. **72:** 949.

INFRARED STUDIES OF TISSUE LIPIDES*

By H. P. Schwarz, L. Dreisbach, R. Childs, and S. V. Mastrangelo
Biochemistry Department, Philadelphia General Hospital, Philadelphia, Pa.

Although some progress has been made in recent years, considerable gaps still exist in our knowledge of the role of complex lipides in the physiological functions or pathological processes of tissues. The main reason for this lack of knowledge lies in difficulties of proper separation and analysis of these complex compounds. Not only do various complex lipides possess similar solubility properties but, when they are present in mixtures, the solubilities of the individual components are quite different from those of the single pure compounds, thus making solvent fractionations extremely difficult or impossible. Because most of the chemical methods of lipide analysis depend upon estimation of hydrolysis products rather than upon determination of intact molecules, chemical characterizations are often doubtful. In addition, these chemical procedures require too large amounts of material to allow complete analysis of such small fractions as may be obtained with more refined separation techniques, for example, chromatography.

Infrared analysis of these tissue constituents, which has been considered as an alternate or supplementary procedure, requires: availability of methods for quantitative analysis of small fractions in the fractional-milligram range; establishment of a catalogue of spectra of pure compounds, isolated from tissue and or synthesized, showing absorption bands sufficiently characteristic to serve for analysis of single components or simple mixtures; standardizations based on such pure compounds; and chromatographic separation of lipides extracted from tissues, yielding either pure single components or simple mixtures suitable for infrared analysis.

Infrared Microanalysis in the Fractional-Milligram Range

Potassium bromide is very transparent to infrared rays and serves as an excellent medium for the preparation of solid suspensions that can be pressed into disks suitable for spectroscopic examination. However, the particle size of the material affects the accuracy of spectroscopic intensity measurements to a considerable degree. Only suspensions with particle diameters as low as about 200 Å., which can be obtained by freeze-drying, behave like true solutions[1] and only disks obtained from such material can be used for quantitative infrared spectroscopy in the fractional-milligram range.[2]

Suspensions of small particles of water-insoluble compounds, including lipides, can be obtained by a two-step freeze-drying procedure developed in this laboratory.[3] In the first step, potassium bromide of small particle size is prepared by freeze-drying of dilute (about 1 per cent) aqueous solutions of potassium bromide of highest purity (obtainable from Merck, Darmstadt, Germany, or Harshaw Chemical Co., Cleveland, Ohio). In the second step,

* The work described in this paper was carried out under Contract AT(30-1)1864 with the United States Atomic Energy Commission.

lipide samples (100 to 250 μg.), dissolved in benzene, benzene:chloroform, or other benzene mixtures, are mixed with 50 mg. of the freshly prepared potassium bromide, and the mixture is freeze-dried again.

The samples are pressed into disks immediately or stored in a vacuum desiccator over P_2O_5 for not longer than 24 hours. The disk-pressing operation, using an evacuable double-plunger steel die of $\frac{1}{4}$ in. diameter, is carried out in an air-conditioned room. The sample taken from the desiccator is quickly crushed in an agate mortar to remove most of the entrapped air. A 50 mg. aliquot of the sample mixture is loaded onto the die. The die is sealed by a slight pressure upon the plunger with a Carver press and is then evacuated to about 1 mm. Hg by means of a mechanical pump. The pressure upon the die is now increased to 8000 lb., and transparent disks of exactly 1 mm. thickness and $\frac{1}{4}$ in. diameter are pressed out within 1 minute.

The disks are weighed, placed in a disk holder, and examined with a Beckman IR2 infrared spectrophotometer that has been modified by installation of a beam-condensing unit between the lamp housing and the liquid-cell compartment.[4] This beam-condensing unit, comprising a system of silver chloride lenses, condenses the infrared beam to the size of the disks inserted at the focal point and thus allows sufficient infrared rays to go through the relatively small specimen.[5] Base-line calculations of the results are carried out in the conventional way.

The accuracy of the spectroscopic measurements was determined by means of duplicate examinations of 45 samples (90 disks) of pure lipides. The over-all standard error of the baseline absorbance measurements was found to be ±0.46 per cent.

Infrared Spectra of Pure Lipides

Naturally occurring and synthesized lipides were studied in solutions, films, and disks, and a catalogue of analytically useful infrared absorption bands was thus obtained. Only a few characteristic spectra of the most important compounds can be given here.

Sphingolipides, such as sphingomyelin, phrenosine, and other cerebrosides, contain a fatty acid amide group, which is absent in all the other lipides. This amide group produces very strong amide I and amide II bands at about 1655 cm.$^{-1}$ (6.04 μ) and 1550 cm.$^{-1}$ (6.44 μ), respectively, that allow distinction of sphingolipides from other complex lipides.[3] Figure 1 illustrates the infrared spectra of sphingomyelin and phrenosine.

If these bands are to be used for analytical purposes it must be taken into consideration, however, that moderate absorptions in the 1650 cm.$^{-1}$ region are also found in cephalin, phosphatidylserine, and acetal lipides. Our own studies of samples of cephalin, the only one of these compounds discussed in this paper, indicate that the very weak absorption at about 1655 cm.$^{-1}$ is caused only to a small extent by vibrations of the cephalin molecule itself, while the greater part of the absorption is produced by traces of water, which absorbs rather strongly in this region. It is believed, therefore, that the amide bands can be used for analysis of sphingolipides in lipide mixtures if necessary precautions are used and/or proper corrections applied.

Both cephalin and lecithin possess fatty acid ester groupings, which are not present in sphingolipides. These ester groups produce very strong C=O bands at about 1740 cm.$^{-1}$ (5.74 μ) in both lecithin and cephalin, thus making them spectroscopically indistinguishable in that region. At lower wave numbers (900 to 1100 cm.$^{-1}$), however, cephalins and lecithins can be distinguished since all the cephalins show a medium strong band at about 1020 cm.$^{-1}$ (9.80 μ), which is absent in lecithins, and all the lecithins show a somewhat weaker

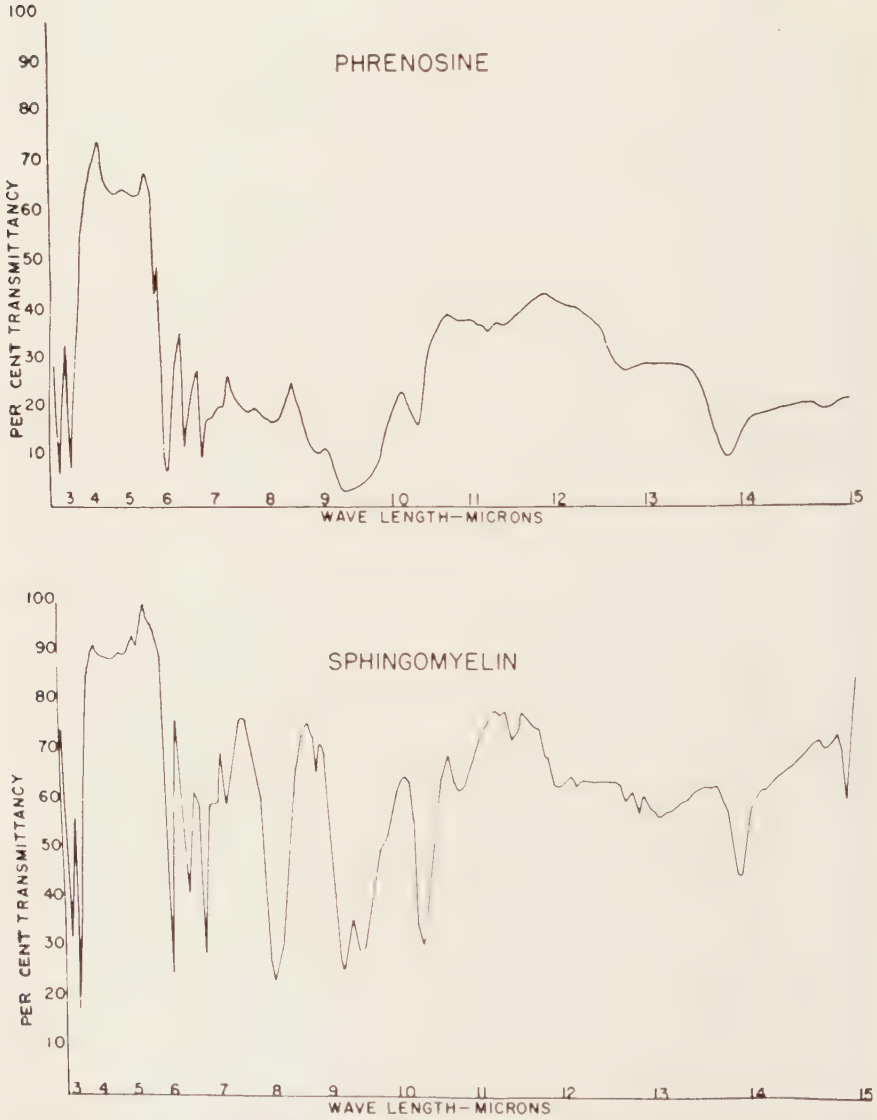


FIGURE 1. Infrared spectra of phrenosine (upper curve) and sphingomyelin (lower curve).

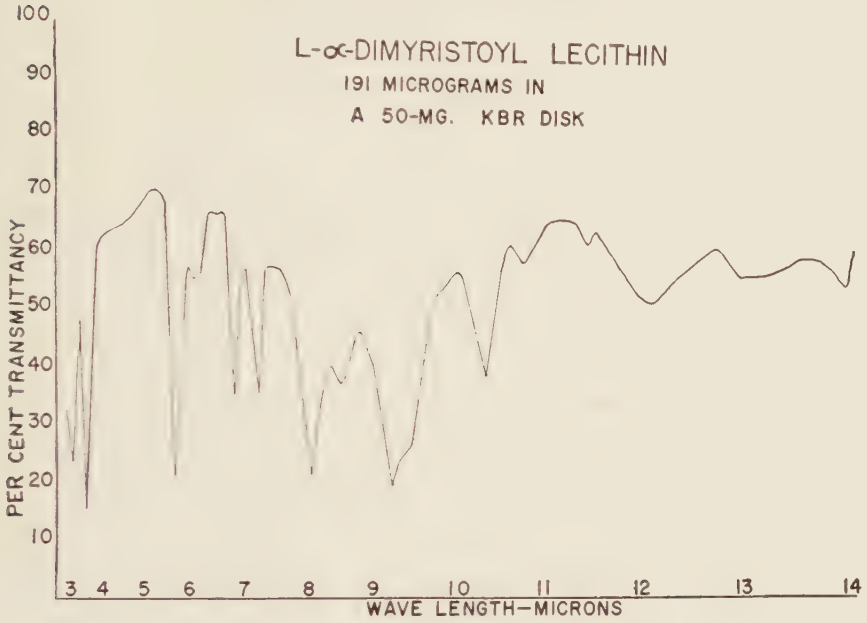


FIGURE 2. Infrared spectrum of synthetic L-α-dimyristoyl lecithin.

band at 970 cm.$^{-1}$ (10.30 μ), which is absent in cephalins. Although no definite assignment of these bands (probably produced by covalent-phosphate vibrations) can be given at present, it is believed that the band at 1020 cm.$^{-1}$ may be valuable for analysis of mixtures of cephalin and lecithin.

FIGURES 2 and 3 show the infrared spectra of a L-α-dimyristoyl lecithin and L-α-dimyristoyl cephalin, which were synthesized by E. Baer. FIGURES 4 and 5 illustrate the spectra of lecithin or cephalin, which were obtained in our own laboratory by chromatographic separation of lipides from the epidermal layers of rabbit skin. Comparison of these spectra (FIGURES 2 to 5) clearly demonstrates the great similarity of the infrared spectra of the synthetic and naturally occurring pure lipides.

STANDARDIZATION OF INFRARED ANALYSIS OF LIPIDES

Standardizations of infrared absorption bands for quantitative analysis are preferably based upon examinations of rather stable synthetic compounds. The spectra of these synthetic compounds must be compared, however, with those of naturally occurring materials in order to correct for possible variations of intensities that may be caused by such factors as different positions of unsaturation. This problem has been studied somewhat more extensively in the standardization of the C=O ester bands of lecithins and cephalins.

STANDARDIZATION OF THE ESTER CARBONYL OF LECITHINS AND CEPHALINS

FIGURE 6 illustrates the comparison of plots of absorbance versus concentration of the C=O ester bands of the synthetic lecithin with those of pure egg

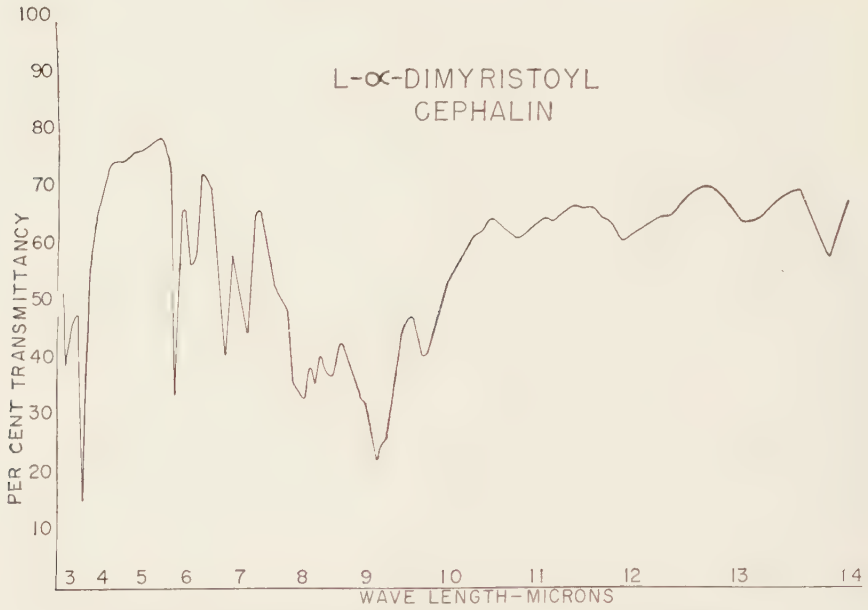


FIGURE 3. Infrared spectrum of synthetic L-α-dimyristoyl cephalin.

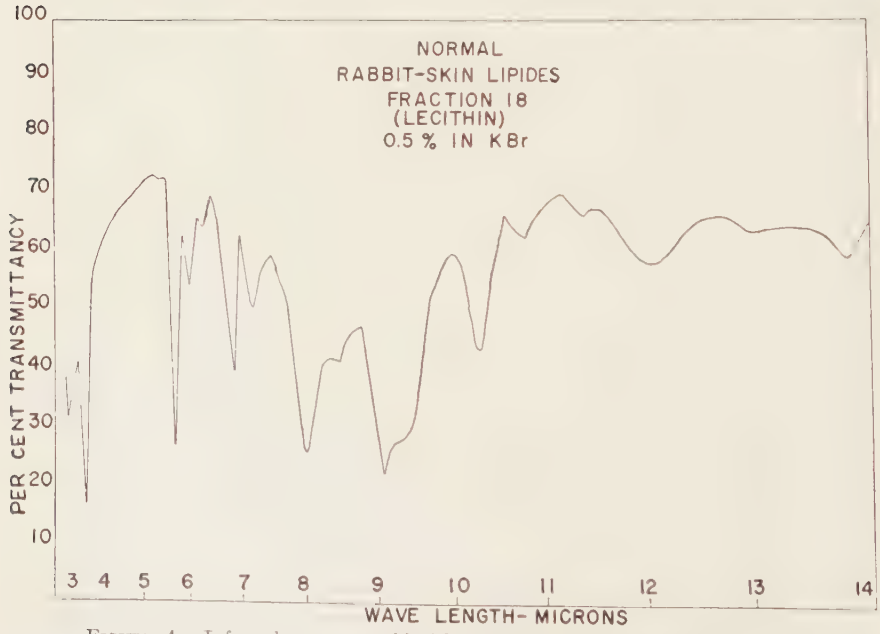


FIGURE 4. Infrared spectrum of lecithin from the epidermis of rabbit skin.

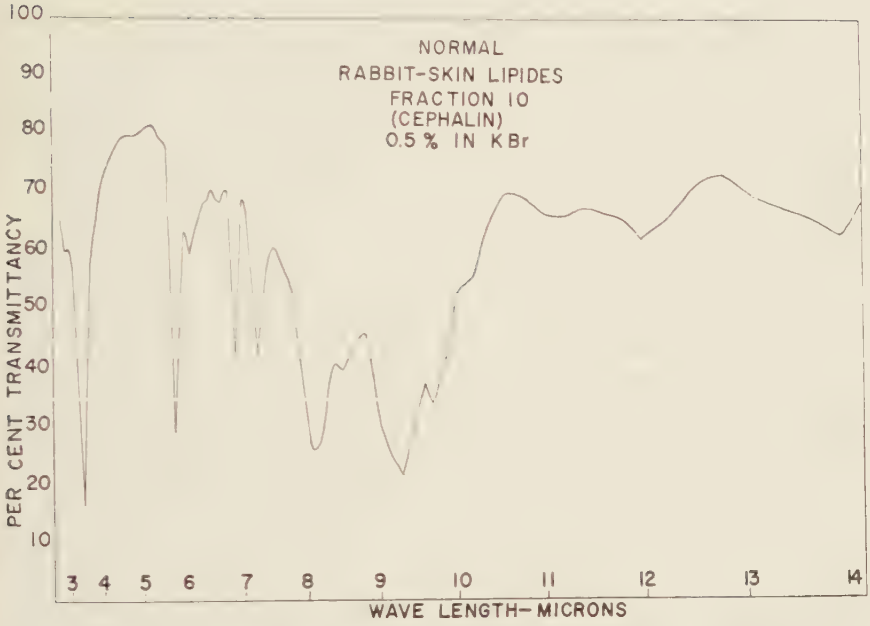


FIGURE 5. Infrared spectrum of cephalin from the epidermis of rabbit skin.

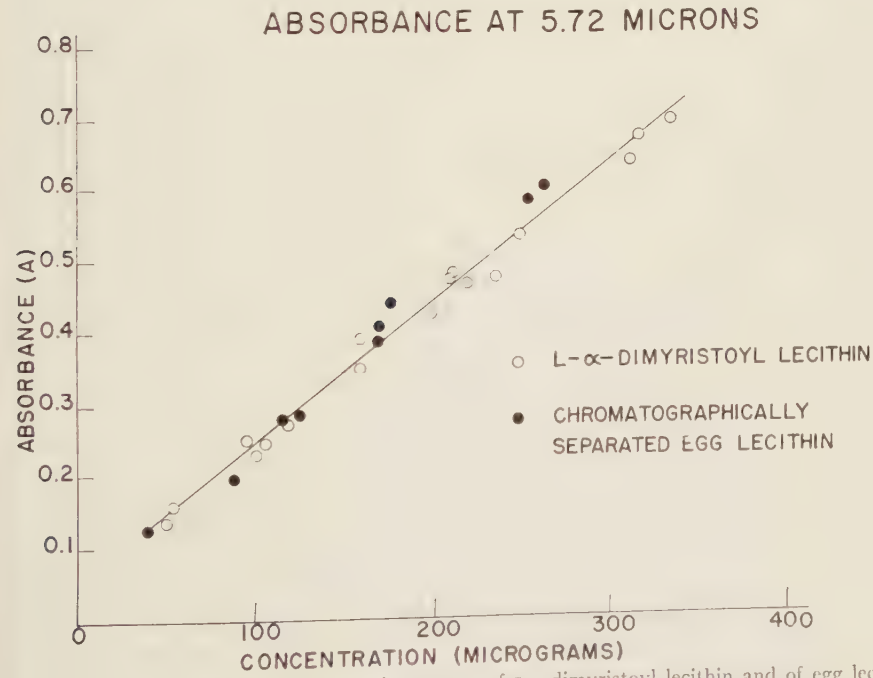


FIGURE 6. Comparison of calibration curves of L-α-dimyristoyl lecithin and of egg lecthin.

lecithin prepared in our laboratory. It may be derived from this figure that the extinction coefficients of these two compounds agree very closely with each other. It must not be concluded from these results, however, that this is a general rule and that the extinction coefficients of all naturally occurring lecithins are identical. Very recent studies of the lecithin of rabbit-skin epidermis indicate that the contrary is true: that is, that definite differences among the extinction coefficients of different materials do exist. It must thus be realized that study of pure naturally occurring materials is an essential prerequisite to infrared analysis of tissue lipides.

Some insight into the probable cause of this variation of the extinction coefficient of the carbonyl bond was obtained by the study of cephalins. It was found that the extinction coefficient of L-α-dimyristoyl cephalin was about the same as that of L-α-dimyristoyl lecithin, but was quite different from the coefficient of the cephalin of rabbit-skin epidermis. The value of the extinction coefficient of this naturally occurring cephalin from rabbit-skin epidermis was, however, almost identical with that of lecithin of the same origin.

The actual values of the average baseline extinction coefficients of both synthetic and naturally occurring lecithins and cephalins are given in TABLE 1.

These data are, of course, too few in number to permit any definite conclusion, but they seem to indicate that lecithins and cephalins that contain similar fatty acid radicals (L-α-dimyristoyl lecithin and L-α-dimyristoyl cephalin) have similar extinction coefficients of their carbonyls. Most naturally occurring lecithins and cephalins, however, probably possess quite different fatty acid groupings[7] and thus would give different extinction coefficient values. The correctness of this assumption is suggested also by a few determinations of carbonyl intensities in simple low-molecular-weight esters, for example, ethyl esters, which show different extinction coefficients in compounds containing different fatty acid groupings.[8] Much more work on the carbonyl intensities of phospholipides is needed. This work will aid, not only in the proper application of infrared spectroscopy to tissue analysis, but also in the elucidation of the complex biological pattern of fatty acids, which are important components of these complex lipides. While these studies were in progress, the extinction coefficients of the synthetic compounds were used for analytical purposes. Comparison of the results thus obtained with those of chemical analysis, which will be given later, shows that correction of the spectroscopic figures will be necessary.

TABLE 1

COMPARISON OF THE EXTINCTION COEFFICIENTS OF THE CARBONYL OF THE SYNTHETIC AND NATURALLY OCCURRING LECITHINS AND CEPHALINS

Source	Compound	100 × E*
Synthetic	L-α-Dimyristoyl lecithin	224
Synthetic	L-α-Dimyristoyl cephalin	231
Rabbit-skin epidermis	Lecithin	172
Rabbit-skin epidermis	Cephalin	162

$$* E = \frac{\text{Baseline absorbance}}{\text{Milligrams of compound in 50 mg. disk}}$$

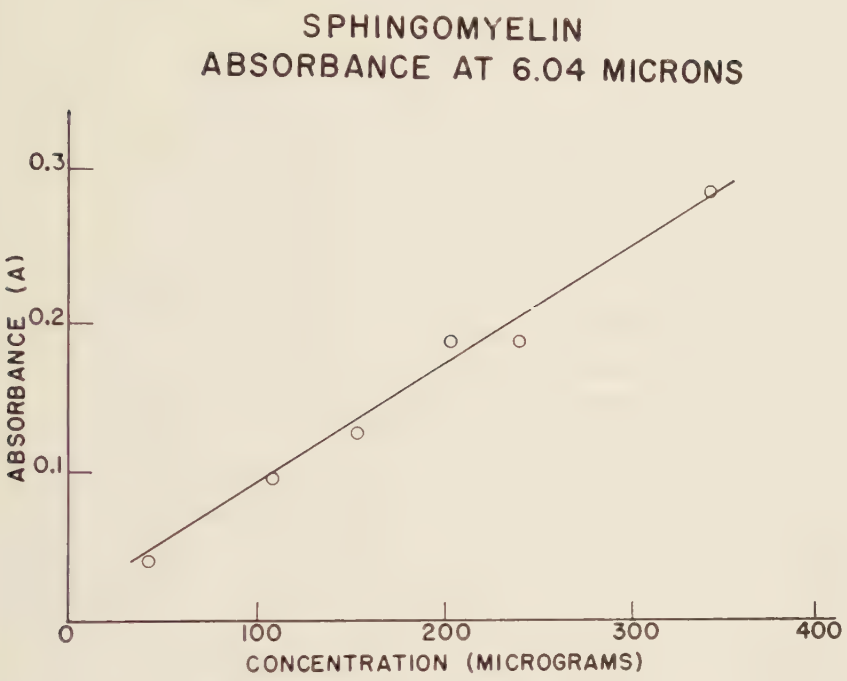


FIGURE 7. Calibration curve of sphingomyelin.

STANDARDIZATION OF THE AMIDE I BAND OF SPHINGOLIPIDES

Determination of the intensity of the amide I band of the sphingolipides was based upon examination of a pure sphingomyelin isolated from spleen (G. Schmidt). Studies of sphingolipides from other sources are still in progress. FIGURE 7 shows a plot of absorbance versus concentration obtained from this sphingomyelin.

INFRARED ANALYSIS OF LIPIDE MIXTURES

Quantitative analysis of lipide mixtures has been tested in simple mixtures that seemed to meet the general analytical requirements, that is, specifically, accurate knowledge of the absorptivities of the individual components and absence of interaction between these components.

Measured amounts of accurately standardized lecithin and sphingomyelin were mixed with weighed amounts of potassium bromide. The disks were prepared and examined as already described. The absorbance values of the ester carbonyl of the lecithin and of the amide I band of sphingomyelin were determined. The conventional calculations were based on the extinction coefficients found in the materials used for making up the mixtures.

TABLE 2, which contains the results of the infrared analysis of these lecithin: sphingomyelin mixtures, shows that the determination can be carried out with an accuracy that could scarcely be attained by microchemical analysis. Simi-

Table 2
Infrared Analysis of Lecithin:Sphingomyelin Mixtures

	Mixture 1		Mixture 2		Mixture 3	
	Calculated	Found	Calculated	Found	Calculated	Found
	μg.	μg.	μg.	μg.	μg.	μg.
Lecithin	404	389	—	—	388	368
Sphingomyelin	200	216	258	256	293	281

lar accuracy has been observed in the infrared analysis of two cephalin:sphingomyelin mixtures.

Chromatographic Separation and Analysis of Lipides

Previous portions of this paper have demonstrated that infrared spectroscopy meets most of the requirements for application to microanalysis of tissues. One additional requirement upon which hinges success or failure, lipide separation, is of equal importance for both infrared and chemical analysis of tissue lipides. Lipide separations must provide the spectroscopist or the chemist with pure single compounds or simple mixtures that are essential as standards.

Chromatography appears to be a promising technique for lipide separation, since Borgström[9] has established the fact that when a chloroform extract of liver, lymph, or intestine is loaded onto a silicic acid column, cholesterol, cholesterol esters, glycerides, and free fatty acids pass through the column upon addition of sufficient chloroform, while the phospholipides are adsorbed and can be eluted quantitatively with methanol. Upon confirmation of this finding with brain lipides, it was decided to test this type of adsorption chromatography for lipide fractionation, but to use gradual elution with chloroform mixtures of increasing methanol content, rather than pure methanol, for the elution of the adsorbed material. Previous experience with reversed-phase partition chromatography and very recent studies of Lea, Rhodes, and Stoll[10] have suggested that such an elution technique may allow recovery of more or less well-separated individual lipides instead of complex lipide mixtures.

It was decided, furthermore, to analyze the chromatographic fractions by infrared spectroscopy and as much as possible by chemical analysis. Infrared data, obtained from the standardizations discussed previously, would be considered as tentative for quantitative purposes and would be corrected as soon as the individual lipides separated could be studied sufficiently (through determining the extinction coefficients of the particular pure tissue lipides). In addition, infrared spectra would be used to indicate the presence of known or unidentified lipides and to supplement and simplify the chemical analysis of numerous small fractions. For the purpose of such simplification, it has been assumed, for example, that the chemical lipide phosphorus content of a fraction showing the infrared spectrum of lecithin is lecithin phosphorus or that the chemical phosphorus of a fraction showing the spectrum of cephalin corresponds to cephalin phosphorus, without the additional determination of other

lipide radicals—choline and ethanolamine, respectively. While at first sight the data based on such assumptions may seem objectionable, they have served well for supplying information about numerous small chromatographic fractions that could not have been analyzed more completely by any other method.

Methods of Chromatography and Combined Infrared and Chemical Analysis of Lipides

Preparation of silicic acid columns. Silicic acid powder (Baker Analyzed Reagent) was washed with chloroform and methanol and then activated in an oven at 110° C. for 24 hours. Twenty-four grams of the freshly activated material was mixed with one fourth of the quantity of filter aid (Johns Manville's Hyflo earth for chromatography washed with distilled water and solvents and dried in a vacuum desiccator over P_2O_5 for 24 hours). The material was then packed into chromatography tubes of 24 mm. diameter. A fresh column prepared in exactly the same way was used for each experiment.

Materials used, loading of the columns, collection of fractions, and preparation of the fractions for analysis. Crude simple lipides, such as egg lecithin (Delta), or mixtures of tissue lipides were used. The former were simply dissolved in a small quantity of chloroform; the latter were extracted from the tissues, freed from nonlipide material,[1] and finally dissolved in the same solvent. The extracts dissolved in chloroform were then loaded onto the columns, a sufficient amount of chloroform (200 to 900 ml., depending on the type of material) was added, and the chloroform fractions were collected upon application of slight nitrogen pressure. The gradual elution of the adsorbed material was carried out by successively passing through the columns chloroform:methanol mixtures of 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 per cent methanol content, and finally pure methanol. As a rule, a total of 23 fractions was collected.

Each of these lipide fractions was concentrated under reduced pressure, then filtered through an ultrafine fritted-glass filter into weighed beakers, and finally evaporated to dryness in a vacuum desiccator over calcium chloride. The filtration is necessary to remove traces of silicic acid, which may pass through any coarser filter fused onto the columns and may thus contaminate the fractions. The dry residue of each fraction was weighed and dissolved in a measured amount of benzene or benzene:methanol. Aliquots of these solutions were used for infrared and chemical analysis.

Analysis of lipide fractions. Quantitative infrared spectrometry of lipide fractions was carried out with the disk method described previously. The analysis of the chloroform fractions was limited to a few estimations of free cholesterol in brain-lipide fractions that were practically free of cholesteryl esters or glycerides. Measurement of the sharp band at about 1060 cm.$^{-1}$ (9.44 μ) allowed accurate estimation of free cholesterol in these lipide fractions. Analysis of the chloroform fractions from other tissues was deferred pending development of further subfractionations.

Each of the 22 fractions eluted with the chloroform:methanol mixtures or with pure methanol was studied spectroscopically. The ester carbonyl band of lecithin or cephalin at about 1740 cm.$^{-1}$ (5.74 μ), the cephalin band at about

1020 cm.$^{-1}$ (9.80 μ), and the amide I band of sphingolipides at about 1655 cm.$^{-1}$ (6.04 μ) were measured. The calculation of the results was based on the standards of L-α-dimyristoyl lecithin, L-α-dimyristoyl cephalin, and sphingomyelin respectively. Small fractions that could not be sufficiently characterized by spectroscopic and/or chemical analysis were designated as unidentified fractions. They were calculated tentatively as lecithin, or cephalin, however, as their elution occurred upon use of almost similar chloroform:methanol mixtures.

Chemical determination of total lipide phosphorus was carried out in all the individual fractions; determination of phosphorus, hexose, total fatty acids, sphingomyelin, and other components was performed as deemed necessary for more complete characterization of the fractions and or as the total amount available permitted. The method of Sperry[12] was used for the microdetermination of total lipide phosphorus. The procedure of Sperry and Brand[13] was applied for determination of hexose (cerebrosides). The other determinations were carried out with the accepted analytical chemical techniques.[14] The chromatograms to be discussed below include values based on total lipide phosphorus or hexose determinations only. The combined use of these chemical and infrared data and the assumptions given in the previous chapter allowed the calculation of the figures as lecithin, cephalin, or cerebrosides, and so forth.

Results of Chromatography and Combined Infrared and Chemical Analysis of Lipides

As examples of the results of the chromatographic separation and analysis of lipides we shall show studies of a crude "single" lipide (egg lecithin) and of tissue lipide mixtures (lipides of the brain or lipides of rabbit-skin epidermis).

Chromatographic separation of egg lecithin. A sample of crude egg lecithin weighing more than 100 mg. was chromatographed and analyzed as described. Determinations of the dry weights of the residues from the fractions indicated a distinct weight peak of 44.9 mg. corresponding to fraction 18. The phospholipide value of this fraction was found to be 41.3 mg., that is, 92 per cent of the total weight of the fraction. The difference (8 per cent) between the weight and phospholipide values was probably due mainly to the water content of the residue since it had been necessary to dry it with calcium chloride. The infrared spectrum of the fraction showed great similarity to the spectrum of lecithin. Study of the intensity of the ester carbonyl band, which has been discussed already (FIGURE 6), further indicated that the extinction coefficient of the ester carbonyl of chromatographically prepared egg lecithin was almost identical with that of synthetic L-α-dimyristoyl lecithin.

It may be pointed out here that chromatography of other material (for example, lipides of rabbit-skin epidermis) regularly showed fraction peaks at about fraction 18 and that these fractions consisted of very pure lecithins (the infrared spectrum of the lecithin from the epidermis of rabbit skin was quite similar to the spectrum of synthetic lecithin, as can be seen by comparison of FIGURE 2 and FIGURE 4).

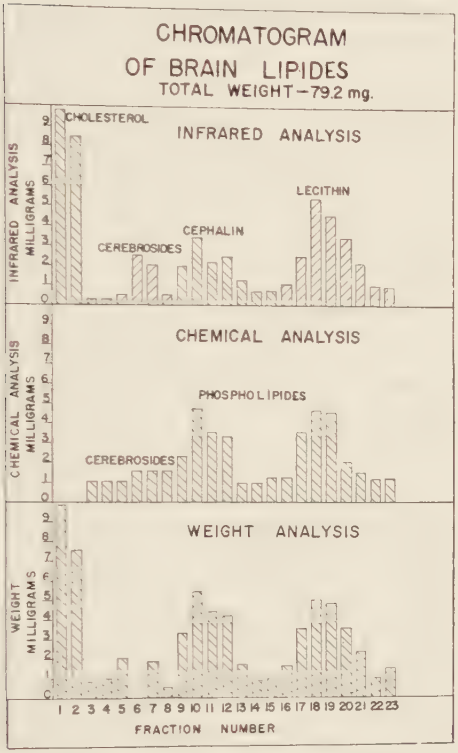


FIGURE 8. Chromatogram of brain lipides. Total weight, 79.2 mg.

Chromatography of brain lipides. A lipide extract of rat brain was chromatographed and analyzed as described. The chromatogram illustrated in FIGURE 8 shows the fraction numbers plotted as abscissas and the corresponding weights and chemical and infrared data (expressed as weight units) plotted as ordinates. The results of the chromatography of brain lipides can be summarized briefly as follows:

(1) The chloroform fractions (1 and 2) contained mainly free cholesterol and were free of phospholipides.

(2) The following 6 fractions (fractions 3 to 8) were also practically free of phospholipides (the total lipide phosphorus value of the 6 fractions was 34 μg.). These 6 fractions consisted almost entirely of galactose-containing sphingolipides—cerebrosides and or gangliosides. The recovery of these sphingolipides was very satisfactory. Comparison of the total hexose content of the unseparated lipide extract with the total hexose content of fractions 3 to 8 of a more recent fractionation showed that 91 per cent of these sphingolipides could be recovered from the column. Further studies will show to what extent cerebrosides can be separated by this method, which may well prove to be one of the simplest and most efficient procedures for the preparation of these complex lipides.

(3) The following fractions (9 to 13) showed most of the described spectroscopic characteristics of the cephalins, with the exception of relatively greater intensity of the amide bands. The chromatogram showed a peak of weight, phospholipide, and spectroscopic cephalin values at fraction 10. This cephalin peak occurred also with great regularity in the chromatograms of the lipides from the rabbit-skin epidermis. However, comparison of the weights and chemical data with the spectroscopic cephalin values shows a distinct difference, which may be caused at least in part by a sphingomyelin-like compound. Determination of the amount of phospholipide that cannot be hydrolyzed easily with alkali agrees with this assumption, as does spectroscopic analysis of the fractions as cephalin: sphingomyelin mixtures. There is reason to believe, however, that plasmalogens may be present in addition.

(4) The following fractions (14 to 22) show the spectroscopic characteristics of the lecithins. The peak of these fractions occurs at about fraction 18. This is the same fraction number that corresponds to the lecithin peaks of the chromatograms of other lipides (egg lipides and lipides of the epidermis of rabbit skin). Differences between spectroscopic and chemical or weight values appearing on the chromatogram are probably caused by deviation of the extinction coefficient of brain lecithin from the extinction coefficient of the synthetic lecithin, which was used for the spectroscopic calculations. These lecithin figures will thus require a correction as soon as the actual extinction coefficients of brain lecithin are established.

The small fraction 23 is not completely identified yet. The infrared spectrum of the fraction showed many bands of lecithins and, in addition, a rather strong band at 1655 $cm.^{-1}$ probably caused by sphingomyelin.

(5) The recovery of the lipide material loaded onto the columns was very high, since the weight recovery frequently approached unity.

Chromatography of phospholipides from normal and irradiated rabbit-skin epidermis. Lipide extracts from nonirradiated or irradiated (24 hours after 700 r of X rays) rabbit-skin epidermis were chromatographed and analyzed as described. The plottings of the chromatograms were revised to give graphic interpretations of the tentative findings rather than the actual results. The fraction numbers were plotted as usual as abscissas, and the tentative chemical or spectroscopic data, expressed in this case as weight percentages of the sum of the fraction weights shown in the chromatograms, were plotted as ordinates. This was thought to be justified since the weight recovery of the material loaded onto the columns was repeatedly found to be close to unity. The two chromatograms are illustrated side by side to allow comparison of lipide distribution (FIGURE 9). The results of these two experiments can be summarized as follows:

(1) The chloroform fractions (fractions 1) were free of lipide phosphorus. Qualitative infrared spectroscopy indicated that the fractions contained mainly glycerides and smaller amounts of sterols and sterol esters. Further examination of these fractions was deferred pending development of subfractionations.

(2) The following 6 fractions (fractions 2 to 8) showed no similarity to the corresponding fractions 2 to 8 of the chromatogram of brain lipides. While these brain-lipide fractions were composed entirely of galactose-containing

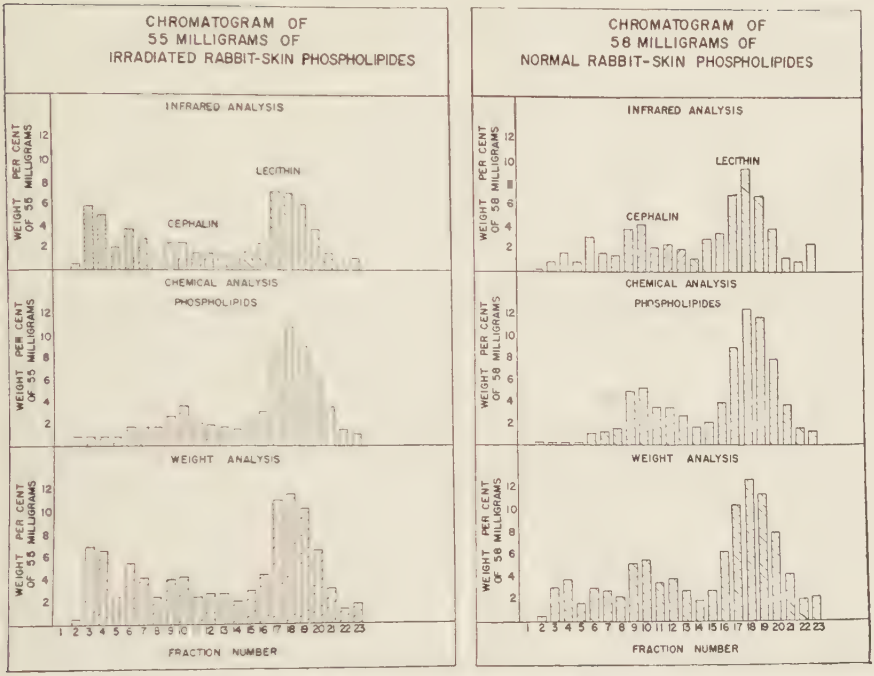


FIGURE 9. Chromatograms of phospholipides from the epidermis of nonirradiated (right chart) and irradiated (left chart) rabbit skin.

sphingolipides, not a trace of galactose was found in the skin lipides. Instead, a rather well-separated unidentified lipide fraction appeared. Identification of this skin lipide fraction must be deferred pending chromatography of a larger amount of material. It may be stated here, however, that this unidentified fraction contained much less lipide phosphorus and more fatty acid ester radicals (measured on the ester carbonyl standard of cephalin) than would correspond to any simple phospholipide.

(3) The following fractions (fractions 9 to 22) were composed of cephalin (fractions 9 to 13), lecithin (fractions 14 to 21), and small amounts of unidentified lipides (fractions 22 and 23). The cephalin and lecithin peaks corresponded to fractions 10 and 18 respectively, and thus showed the same position on these chromatograms as they did on the previously discussed chromatograms of lipides of other origin (egg lipides and brain lipides). The cephalins and lecithins from the rabbit-skin epidermis exhibited a high degree of purity, which has already been demonstrated by the infrared spectra of fractions 10 and 18 (FIGURES 4 and 5).

The extinction coefficient of these lecithins and cephalins (TABLE 2), however, deviated significantly from the extinction coefficients of the synthetic compounds upon which the calculations of the infrared data in the chromatogram were based. This part of the results given in the chromatogram would thus require a correction if they were to be used for quantitative purposes.

The small unidentified fractions (*22* and *23*) gave spectra quite similar to those from the unidentified fraction *22* of the brain-lipide fractionation and thus will require further elucidation.

Conclusions

(1) The potassium bromide disk technique has been adapted for quantitative infrared analysis of lipides in the fractional milligram range.

(2) Methods for infrared microdetermination of cephalin, lecithin, and sphingolipides are described. The accuracy of infrared analysis of single lipides and lipide mixtures is demonstrated.

(3) Proper application of these infrared methods for routine analysis of tissue lipides depends greatly on accurate studies of the intensities of the characteristic absorption bands to be used for analytical purposes. The extinction coefficients of such absorption bands as the ester carbonyl of lecithin or cephalin may vary significantly in naturally occurring lipides of different origin, which probably contain fatty acids of different chain length and unsaturation. Fractionation of tissue lipides is thus necessary for preparation of pure compounds to be used as spectroscopic standards.

(4) Adsorption chromatography, employing silicic acid columns and gradual elution of the adsorbed material with chloroform:methanol mixtures of increasing methanol content, was used for lipide separation. The numerous small chromatographic fractions were analyzed by combined infrared and analytical chemical techniques, which proved valuable for checking the purity of known lipide fractions as well as for the detection of unknown lipides.

(5) Highly purified lecithins, cephalins, and galactose-containing sphingolipides were obtained, and a number of unidentified lipides were detected with the described methods.

(6) It is believed that further development of lipide fractionation techniques is of utmost importance, not only for better characterization of lipides, but also for the exclusive use of highly accurate and specific infrared methods in the elucidation of the complex problems of lipide chemistry.

References

1. Schiedt, U. & H. Reinwein. 1952. Z. Naturforsch. **7b:** 270.
2. Schwarz, H. P., R. Childs, S. V. R. Mastrangelo & L. Dreisbach. 1956. Science. **123:** 328.
3. Schwarz, H. P., R. Childs, S. V. R. Mastrangelo & L. Dreisbach. 1955. Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. Abstr. 155.
4. Anderson, D. H. & N. B. Woodall. 1953. Anal. Chem. **25:** 1906.
5. Toribara, T. Y. 1954. Anal. Chem. **26:** 2003.
6. Schwarz, H. P., H. E. Riggs, C. Glick, J. McGrath, W. Cameron, E. Beyer, E. Bew, Jr. & R. Childs. 1952. Proc. Soc. Exptl. Biol. Med. **80:** 467.
7. Klenk, E. & P. Bohm. 1951. Z. physiol. Chem. Hoppe-Seyler's **288:** 98.
8. Hampton, R. R. & J. E. Newell. 1949. Anal Chem. **21:** 914.
9. Borgström, B. 1952. Acta Physiol. Scand. **25:** 101.
10. Lea, C. H., D. N. Rhodes & R. D. Stoll. 1955. Biochem. J. **60:** 353.
11. Folch, J., L. Ascoli, M. Lees, J. A. Meth & F. N. LeBaron. 1951. J. Biol. Chem. **191:** 833.
12. Sperry, W. M. 1942. Ind. Eng. Chem. Anal. Ed. **14:** 88.
13. Sperry, W. M. & F. C. Brand. 1941. J. Biol. Chem. **141:** 545.
14. Man, E. B. & E. F. Gildea. 1932. J. Biol. Chem. **99:** 43.

INFRARED SPECTROSCOPY OF SERUM LIPIDES*

By Norman K. Freeman

Donner Laboratory of Biophysics and Medical Physics, University of California, Berkeley, Calif.

The study of infrared absorption by substances in aqueous solution is attended by well-known limitations. Among these restrictions is the requirement of a very thin absorbing layer and, consequently, a relatively high concentration of the dissolved substance, usually at least 5 to 20 per cent. Furthermore, even in very thin layers, there are spectral regions near 3300 cm.$^{-1}$ and 1650 cm.$^{-1}$ that are totally obscured by intense absorption bands of water. In view of these factors the possibility of making useful interpretations from the infrared spectrum of serum itself seems remote. Protein, the principal solute in serum, has a concentration of about 7 per cent, and the total lipide content is only about 0.5 to 1 per cent. A spectrum of serum recently published by Potts and Wright[1] reveals only very weak absorption bands in the 1500 to 1000 cm.$^{-1}$ region, most of which are probably attributable to protein.

If the serum is dehydrated to form a dried film, its spectrum is still essentially that of the proteins, owing to their presence in approximately tenfold excess over the amount of lipide. Even the strongest lipide absorption bands, at 2940 cm.$^{-1}$ and 1720 cm.$^{-1}$ (3.4 μ and 5.8 μ), are effectively masked by nearby protein bands. In the examples of dried-serum spectra that have been reported by Blout and Mellors[2] and by Woernley[3] these principal lipide bands are not evident. It is clear, therefore, that some sort of separation or enrichment of the lipides is necessary before they can be identified spectroscopically.

Lipoproteins

The next step that might be taken, short of complete separation of the lipides by extraction, is to concentrate them in the form of lipoproteins. These are the well-known lipide-protein complexes that provide the means of solubilizing the lipides in an aqueous medium. Since the lipoproteins are important entities in the lipide transport system and have been correlated statistically with the development of atherosclerosis and coronary artery disease,[4] they have been a subject of continuing study in our laboratory by a variety of means. FIGURE 1 shows the infrared spectra of some dried lipoprotein films, which have been discussed in some detail elsewhere.[5] The sequence of curves corresponds to a series of lipoprotein classes of increasing lipide content that are separable by ultracentrifugal techniques. This change in composition — from about 30 to 95 per cent lipide — is reflected in the increasing intensity of the 2940 cm.$^{-1}$ and 1720 cm.$^{-1}$ bands relative to the principal protein bands at 3300 cm.$^{-1}$ (3 μ), 1640 cm.$^{-1}$ (6.1 μ), and 1540 cm.$^{-1}$ (6.5 μ). These spectral features provide a rough index of the lipide-protein ratio, and a certain amount of qualitative information can be derived from the pattern between 900 and 1400 cm.$^{-1}$ Such dried-film preparations are not conducive to good quantita-

* The work described in this paper was supported by the United States Atomic Energy Commission.

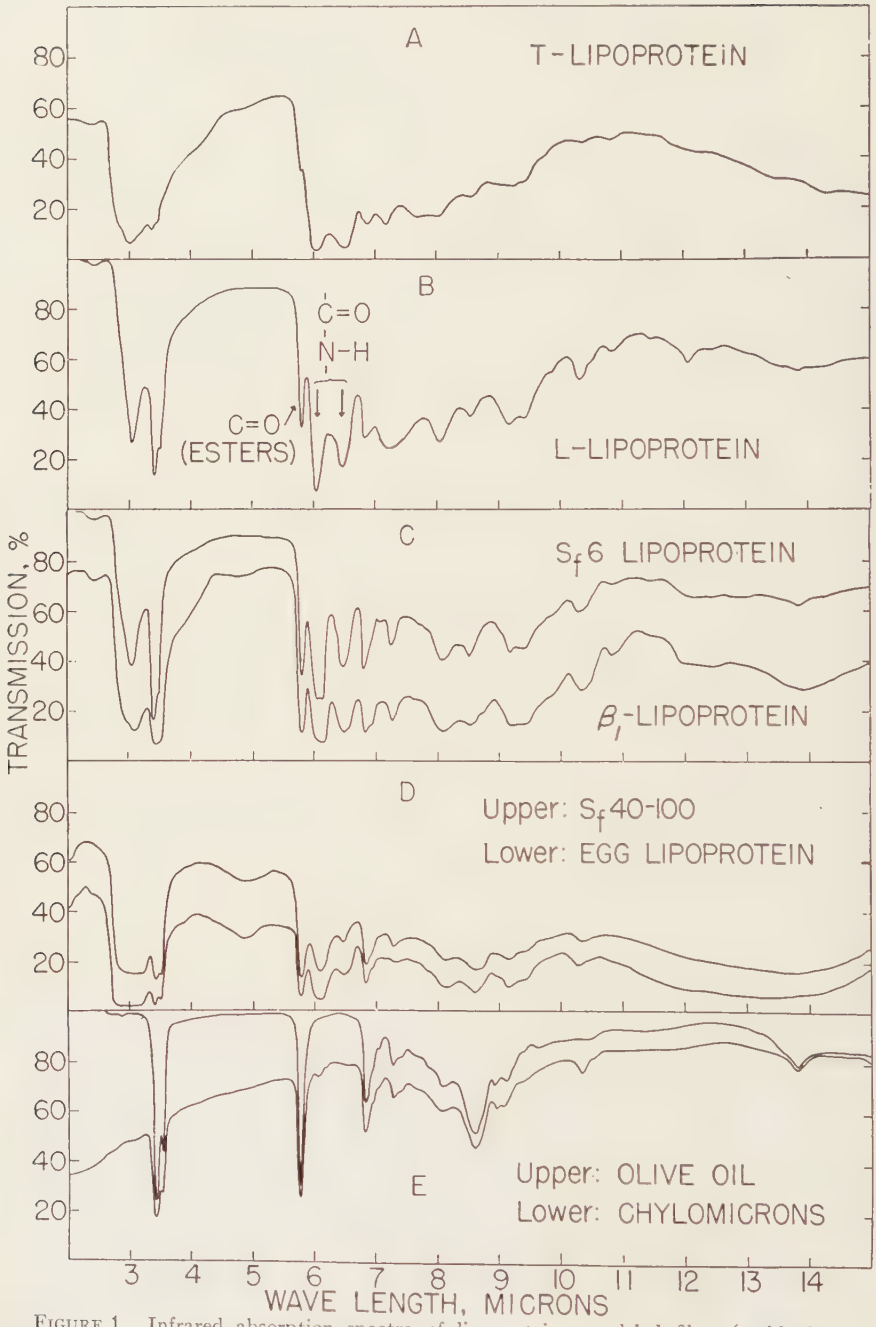


FIGURE 1. Infrared absorption spectra of lipoproteins as dried films (residual water present in D). A → E, increasing lipide content.[5] (Reproduced by permission of *The Journal of Biological Chemistry*.)

tive measurements; however, by the application of a simple solvent-extraction procedure to these films a crude quantitative analysis was developed. Because of the shortcomings inherent in this method, it proved to be of limited applicability. A more promising method for studying the spectra of intact lipoproteins is to freeze-dry them with potassium bromide and press the powdered mixture into a pellet. We have obtained good spectra by this technique, but have not fully evaluated its quantitative aspects.

Because of the complications arising from the presence of protein, we have devoted considerably more attention to the lipides themselves, for which quantitative measurements are more feasible since they can be dissolved in appropriate organic solvents for spectrophotometric study.

Combination of Infrared Spectroscopy with Chromatography

Principal lipide classes. From our experience in the spectroscopic study of lipoproteins, it seemed that a breakdown of the total lipide mixture into its constituent classes would be desirable. A significant point in this connection is the close similarity of the spectra of cholesteryl esters and the spectra of triglycerides. While the two groups are readily identifiable when separated, they are difficult to distinguish or analyze in mixtures containing both. To perform a separation, we have adopted (with minor modifications) the chromatographic scheme described by Borgström.[6] A complete separation of individual lipide types is not achieved, but two of the major classes are separated from the remainder. By combining this separation technique with infrared spectrophotometric measurements of the fractions obtained, one has what might be regarded as a basic system of lipide analysis. The procedure, as we have used it, is to be published in detail elsewhere,[7] but it may be outlined briefly here. The total lipide mixture extracted from 1 ml. of serum (5 to 10 mg. of lipide) is dissolved in a minimum amount of hexane and is put on a 0.25 gm. column of activated silicic acid-Celite (2:1). Three fractions are eluted, as outlined in TABLE 1. Each fraction is evaporated to dryness, redissolved in a small measured volume of carbon disulfide, and its infrared spectrum is obtained. The spectra of a typical set of fractions are shown in FIGURE 2. From measurements of absorption at appropriately selected band peaks, together with the necessary calibration data determined from reference materials, it is possible to calculate the concentrations of the various components (an average molecular

TABLE 1

SEPARATION OF LIPIDE CLASSES BY ADSORPTION ON SILICIC ACID-CELITE
(5 to 10 mg. sample, 0.25 gm. column)

Fraction	Eluting solvent	Eluted components	Calibration wave length
I	$CHCl_3$-hexane (1:19), 8 ml.	Cholesteryl esters	5.8 μ
II	$CHCl_3$, 8 ml.	Glycerides	5.76 μ
		Unesterified cholesterol	9.5 μ
		Unesterified fatty acids	5.84 μ
III	Methanol, 8 ml.	Phosphatides	9.4 μ

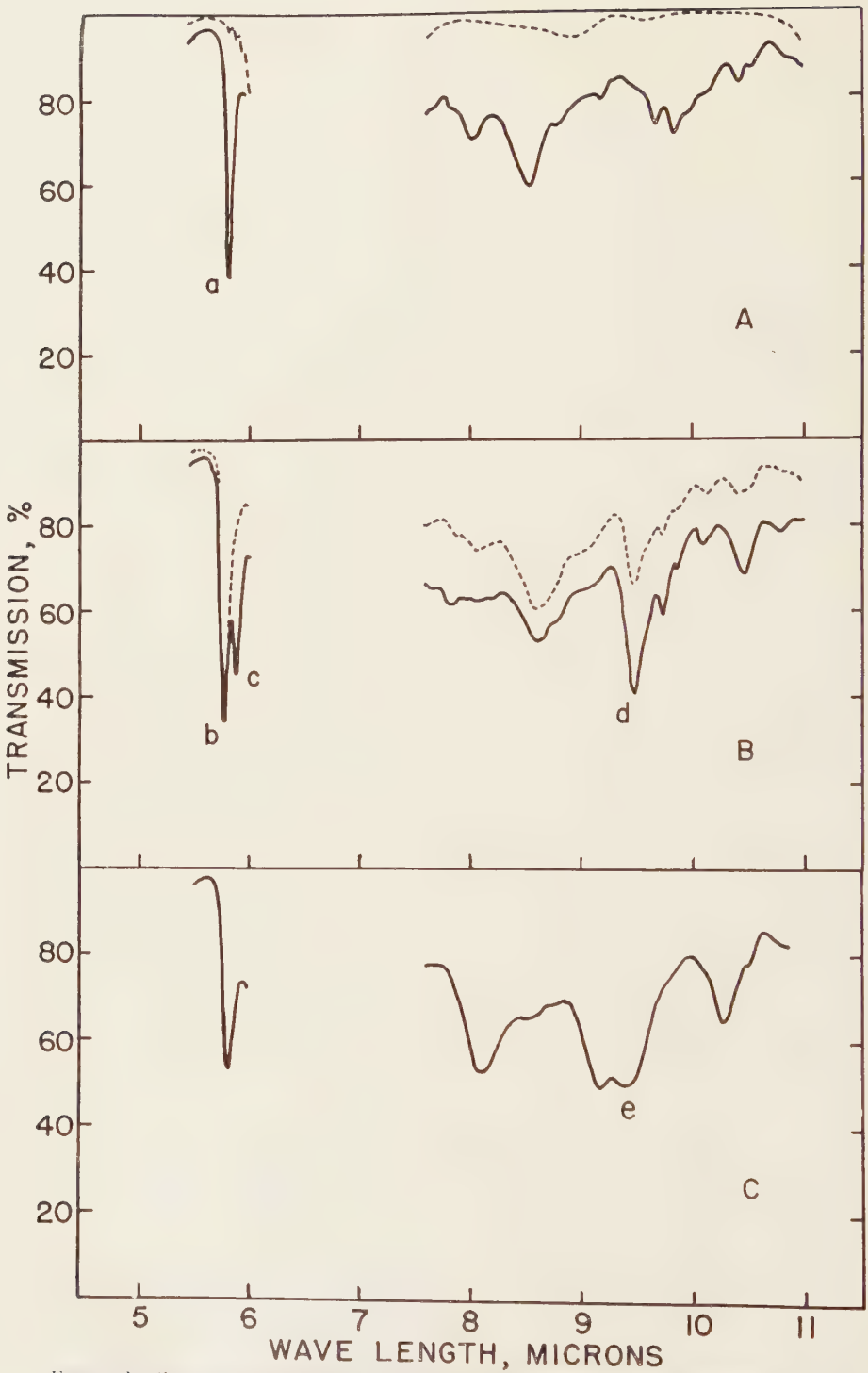


FIGURE 2. Spectra of CS_2 solution of lipide fractions obtained by chromatography: (A) cholesteryl esters; (B) glycerides, fatty acids, cholesterol; (C) phosphatides. Calibrated bands are indicated by small letters.[7] (Reproduced by permission of *The Journal of Biological Chemistry*.)

weight of fatty acids is arbitrarily taken as that of oleic acid). In Fractions I and III only one absorption measurement need be made. For cholesteryl esters the carbonyl band at 1720 cm.$^{-1}$ (5.8 μ) is used; for total phosphatides a band at 1065 cm.$^{-1}$ (9.4 μ) characteristic of the P-O-C group in phosphoric esters has been found satisfactory. The mixture comprising Fraction II requires the treatment of multicomponent analysis. It is a fortunate circumstance that unesterified cholesterol contributes essentially no absorption in the carbonyl region near 1720 cm.$^{-1}$; hence a two-component system of glycerides and fatty acids can be established using ester absorption at 1735 cm.$^{-1}$ (5.76 μ) and carboxyl absorption at 1712 cm.$^{-1}$ (5.84 μ) as the basis. Calculated concentrations of these two components are then used for determining corrections to the measured absorbance at 1050 cm.$^{-1}$ Cholesterol is calculated from this corrected value.

The information obtained by this procedure is equivalent to a description of the lipide composition that would otherwise require chemical determinations of total cholesterol, unesterified cholesterol, total fatty acids, unesterified fatty acids, and lipide phosphorus. The accuracy of the method in its present state of development is of the order of ± 10 per cent (except unesterified cholesterol, for which the accuracy suffers in consequence of its low concentration, low absorptivity, and the necessity of applying a double correction to the measurement). Since the method is nondestructive, the fractions are recoverable for further investigation if desired. The total sample requirement is about 5 mg. for an absorption cell 5 × 21 mm. in cross section that has been used in a Baird Associates double-beam spectrophotometer. With smaller microcells and the appropriate instruments to accommodate them, samples weighing less than 1 mg. could presumably be analyzed.

The lipide compositions of various lipoprotein classes in human serum have been studied by this method and reported.[8]

Considering this combination of chromatography and infrared analysis as a basic scheme, some extensions of it are being explored with the purpose of further subdividing the three fractions obtained. These investigations have not progressed very far, and the brief descriptions that follow are mainly indicative of the approaches being used and the manner in which infrared spectrometry is involved.

Cholesteryl esters. The first illustration is an attempt that is being made to develop a chromatographic separation of the various cholesteryl esters. In doing this we have coupled the column directly to an infrared spectrometer; that is, the effluent is allowed to flow through an infrared absorption cell *in situ* and the absorption at a fixed wave-length setting of 3.4 μ (2940 cm.$^{-1}$) is recorded as a function of the volume that has passed through the column. The initial trials of this experimental method have been made with columns containing 0.5 gm. or 1 gm. of silicic acid-Celite (2:1), from which 1 to 2 mg. of cholesteryl esters were eluted with CCl_4. The spectrometer was a Perkin-Elmer single-beam double-pass (Model 112) instrument with a sodium chloride prism. The Perkin-Elmer microcell used had a path length of 3 mm. and a volume of 0.07 cm.^{3} Elution curves obtained in this way for 1 mg. samples of cholesteryl laurate and serum cholesteryl esters are shown in FIGURE 3. The

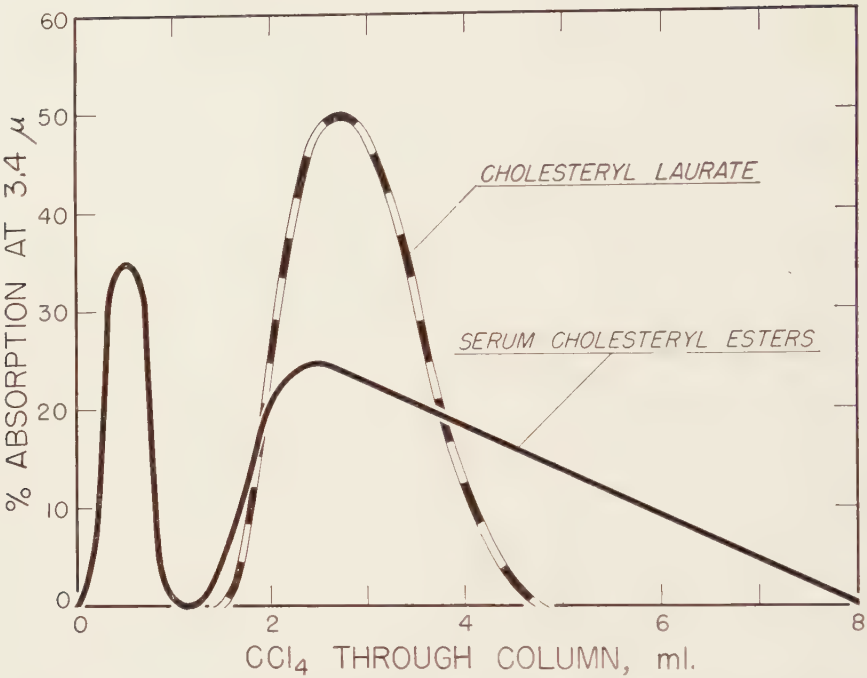


FIGURE 3. Elution curves of cholesteryl laurate and serum cholesteryl esters from silicic acid-Celite (2:1) by CCl_4. The ordinate is the spectrophotometric absorption measurement of the effluent in a 3 mm. microcell (the initial peak in the serum ester curve may be a hydrocarbon contaminant).

origin of the initial peak in the serum ester curve is uncertain. It may represent a hydrocarbon contaminant, but this needs to be verified. While no definite peaks have been resolved for the serum esters, there appears to be a retardation of these as compared with laurate. This effect is presumably related to the difference in fatty acid unsaturation. It does not seem at all unlikely that, by varying the type of column, the adsorbent, and or the operating conditions, some resolution of either the cholesteryl esters themselves or the methyl esters derived from them can be achieved. For exploring these variations, infrared absorption measurement provides a neat and convenient tool. This system is not universally applicable, however, since only a limited number of nonpolar solvents, such as carbon tetrachloride and carbon disulfide, can be used at the path lengths required for good sensitivity.

Phosphatides. Further chromatography of the phosphatide fraction has also been undertaken. Since mixtures of chloroform and methanol are being used as eluting solvents, direct infrared measurement of the column effluent as described above is not feasible. Instead, we are employing the straightforward procedure of collecting fractions, with subsequent evaporation of each to dryness, and redissolution in an appropriate solvent for infrared examination. Starting with the work of Lea, Rhodes, and Stoll,[9] who used silicic acid columns to separate the cephalins and lecithins of egg phosphatides, we have sought

TABLE 2
FRACTIONATION OF TOTAL METHANOL ELUATE

Fraction	Eluting solvent	Eluted components	Approximate percentage of total fraction
IIIa	Acetone	Unidentified nonphosphatide	5–10%
IIIb	15% Methanol in $CHCl_3$	Cephalins	5–10%
IIIc	60% Methanol in $CHCl_3$	Lecithins	65–75%
		Sphingomyelins	15–20%
IIId	Methanol	Unidentified nonphosphatide	1–2%

not only to reproduce this result for serum phosphatides, but also to find conditions for separating the sphingomyelins from lecithins. There does appear to be a separable fraction having the characteristics of cephalin but so far, using various ratios of chloroform and methanol, we have not accomplished the second objective. The sphingomyelins trail the lecithins, but there is considerable overlapping. The present status of the subfractionation of the material ordinarily eluted by methanol is summarized in TABLE 2. Other known phosphatide constituents, such as plasmalogens and phosphatidyl serine, are believed to be minor constituents not likely to be identifiable in the presence of the major ones. The spectra of the principal phosphatide types are shown in FIGURE 4.

Even if no way of cleanly separating lecithin from sphingomyelin is found, we are in a better position to analyze this subfraction as a two-component mixture than we would be to analyze the total fraction containing cephalin and nonphosphatide impurities. The possibility of such a two-component analysis, based on the 1720 $cm.^{-1}$ (5.8 μ) band of lecithin and the 1640 $cm.^{-1}$ (or possibly 1540 $cm.^{-1}$) band of sphingomyelin, has been pointed out by Marinetti and Stotz.[10] While we are not too confident of our calibration data for sphingomyelin at present, we have set up this procedure and used it for estimating the compositions of chromatographic fractions obtained in developing the separation method, and also in aortic tissue samples in which the sphingomyelin content differs from that in blood.

The cephalin fraction has the correct spectral characteristics, notably the absence of the band at 970 $cm.^{-1}$ (10.3 μ) that is exhibited by both lecithin and sphingomyelin. This material contains approximately 4 per cent phosphorus, and it elutes under the same conditions as egg cephalin.

Little attention has been devoted to the impurity fractions as yet, except to establish the absence of phosphorus and to consider their effects on the analytical results. For the total phosphatide measurement the effects are very small.

Analysis of Total Serum Lipide Extract without Fractionation

While proceeding in the direction of complete fractionation as a desirable and, in a certain basic sense, necessary approach to lipide analysis, one still seeks simplified methods that serve many routine purposes, even if the results obtained are limited in scope or accuracy. In this light the possibility of de-

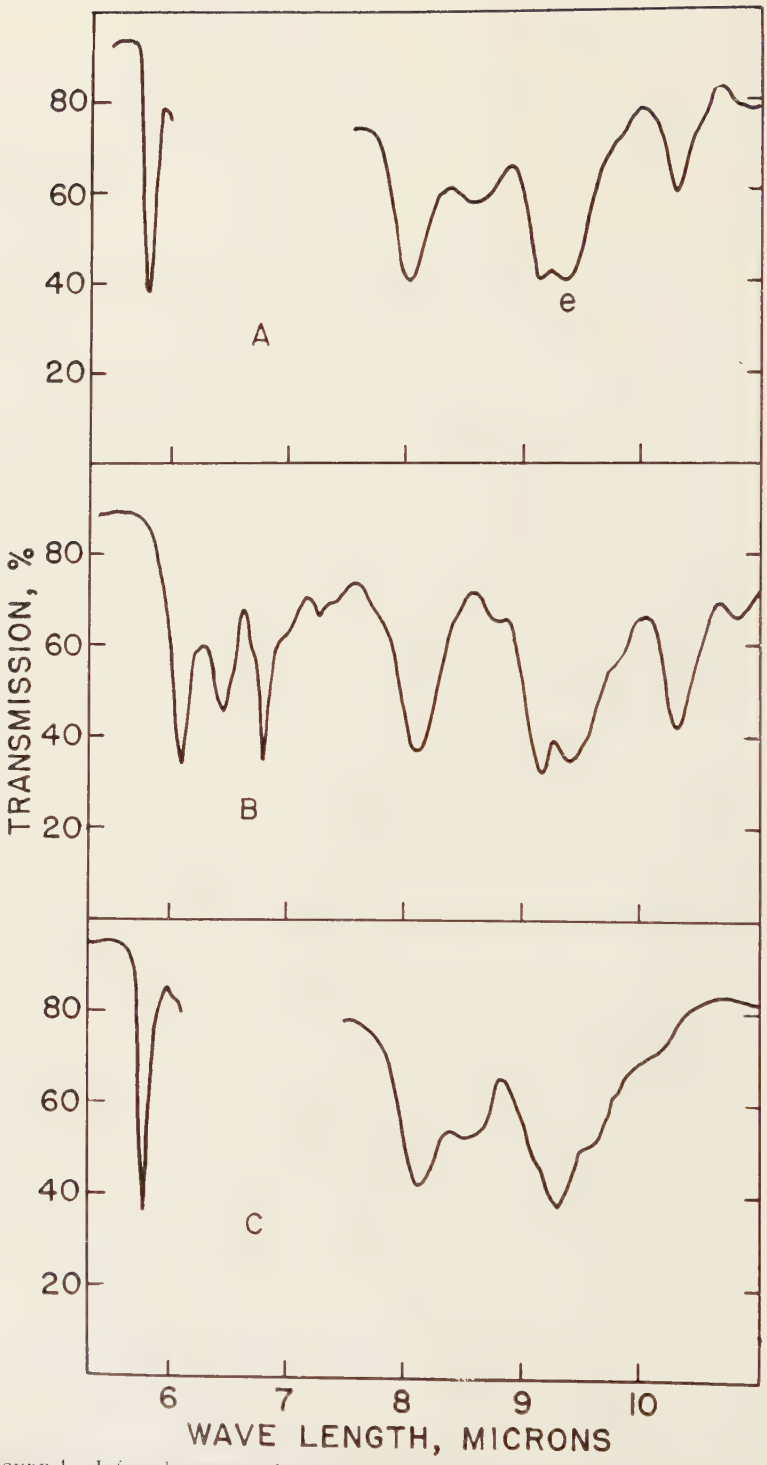


FIGURE 4. Infrared spectra of principal types of phosphatides: (A) lecithin in CS_2 solution; (B) sphingomyelin, solid film; (C) cephalin in CS_2 solution.[7] (Reproduced by permission of *The Journal of Biological Chemistry*.)

riving useful information from the infrared spectrum of the total serum lipide extract should be considered. Renkonen and Koulumies[11] compared the spectra of total lipide extracts (CCl_4 solutions) with those of glycerides, lecithin, sphingomyelin, and cholesterol, but they failed to include cholesteryl esters among their reference compounds. They attempted to make some comparisons between the total lipide spectra of diabetics and those of normal persons, principally in terms of numerical absorbance values calculated at various wave lengths. Some limited and tentative interpretations were offered concerning the differences observed for certain absorption bands.

Knowledge of the constituents and the character of their spectra is prerequisite to the formulation of a satisfactory multicomponent analysis based on infrared spectrophotometry. For serum lipides it is pertinent to realize that some methods of extraction in common use leave nonlipide impurities such as urea and amino acids in the final mixture. It is therefore necessary either to eliminate such impurities or to know their spectra and include them as components. The first course is certainly preferable, and we have presumed that the extraction procedure described by Sperry[12] is satisfactory with respect to both completeness of lipide extraction and the elimination of nonlipide impurities.

For a known set of components, then, there should exist for each component a reasonably strong band at some spectral position where the absorption by all other components is relatively weak. If the spectra of the various serum lipide types are examined with respect to this requirement, it is seen that the condition is not fulfilled. It has already been pointed out that the principal absorption bands of cholesteryl esters are very close to those of the glycerides. Also, the (otherwise) most suitable band of cholesterol is overlapped by a somewhat stronger band of the phosphatides. Thus it seems that a straightforward conventional multicomponent analysis is unlikely to be workable.

A typical spectrum of total serum lipide extract (in CCl_4 solution) is shown in FIGURE 5. The molecular origins of the principal absorption bands are indicated, and they suggest a possible analysis on a basis that resembles the classical chemical description. That is to say, it appears potentially feasible to determine the composition in terms of total esterified fatty acids, total cholesterol, and total phosphatides. Since phosphatides contain esterified fatty acids, we have defined an independent component that may be called "residual phosphatides": this is simply what is left if the esterified fatty acids are taken away. In the usual way, then, equations can be written to express the measured absorbances at designated wave lengths as sums of the component absorbances.

$$A^{5.8\mu} = (\alpha^{5.8}_{EFA} \times C_{EFA}) + (\alpha^{5.8}_{TC} \times C_{TC}) + (\alpha^{5.8}_{RP} \times C_{RP})$$

$$A^{7.25\mu} = (\alpha^{7.25}_{EFA} \times C_{EFA}) + (\alpha^{7.25}_{TC} \times C_{TC}) + (\alpha^{7.25}_{RP} \times C_{RP})$$

$$A^{9.15\mu} = (\alpha^{9.15}_{EFA} \times C_{EFA}) + (\alpha^{9.15}_{TC} \times C_{TC}) + (\alpha^{9.15}_{RP} \times C_{RP})$$

where EFA = esterified fatty acids; TC = total cholesterol; RP = residual phosphatides (total phosphatides − EFA in phosphatides); α = absorbance/gm./l., in the particular cell used; and C = concentration, gm./l. The equations form a linear simultaneous set, to be solved for concentrations. The α's in

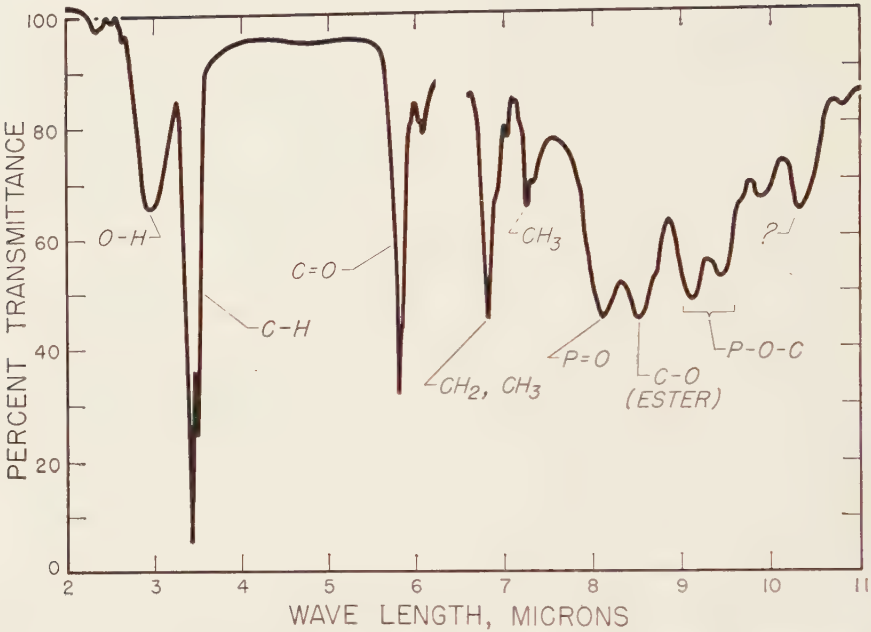


FIGURE 5. Infrared spectrum of total lipide extract from 1 ml. of serum. Approximately 10 mg./ml. in CCl_4 (0.9 mm. cell).

these equations might be called "mean partial absorptivities*." The first problem is to assign values to them from the calibration data obtained with reference compounds. If a reasonable set of constants can be found, then the results can be tested empirically.

The first simplification may be made by assigning

$$\alpha_{TC}^{5.8} = 0; \qquad \alpha_{RP}^{5.8} = 0$$

This states the assumption that the 5.8 μ (1720 cm. $^{-1}$) band is exclusively due to ester carbonyl groups. The measurement of esterified fatty acids from this single band absorption therefore depends on the validity of an average $\alpha_{EFA}^{5.8}$. To evaluate this constant we have expressed the absorptivity of each ester type as absorbance per gram per liter of esterified fatty acids (of some selected average molecular weight). Actually, the peak absorptions for the various esters do not occur at precisely the same wave length. The central peak (phosphatides) is very close to 1725 cm. $^{-1}$ (5.80 μ) and, in the terms just cited, it is the weakest of the three. The other bands, although they are stronger at their peaks, are sharp enough to show that their estimated absorptivities are smaller than the absorptivity of the phosphatides at 1725 cm. $^{-1}$. Calibration data at that wave number in a cell 0.9 mm. thick give "partial absorptivities" of 0.07, 0.12, and 0.10 for glycerides, phosphatides, and cholesteryl esters,

* This usage of the term absorptivity is not strictly correct, since all of the α's include the cell thickness as a constant multiplier.

respectively. Since all three classes make substantial contributions, the approximate mean value, 0.10, has been tentatively adopted for trials.

The determination of the other α's can be illustrated in the following way. At 1380 cm.$^{-1}$ (7.25 μ) the absorptivity of fat (olive oil) can be converted to the desired value by multiplying it by the weight ratio of fat to esterified fatty acids, thus:

$$\alpha_{EFA}^{7.25} = \frac{884}{846} \times \alpha_{\text{Fat}}^{7.25}$$

If the value obtained, 0.0181, is to be valid, we should be able to combine it with the value of cholesteryl ester absorptivity at this wave length and calculate the correct value for unesterified cholesterol. The assumption being tested is that

$$\alpha_{CE}^{7.25} = (\alpha_{EFA}^{7.25} \times \text{wt. fraction } EFA) + (\alpha_{UC}^{7.25} \times \text{wt. fraction } UC)$$

α_{UC} calculated from this relationship is 0.0272, compared with the measured value of 0.028. The degree of self-consistency among fat, cholesteryl esters, and cholesterol at this wave length appears to be fairly good.

Lecithin may be used to evaluate $\alpha_{RP}^{7.25}$ by using the value for $\alpha_{EFA}^{7.25}$ determined from fat, combined with the measured lecithin absorptivity.

$\alpha_{RP}^{9.15}$ is determined by subtracting an esterified fatty acid contribution from a weighted absorptivity of lecithin plus sphingomyelin. The esterified fatty acid contribution evaluated from fat does not agree too well with that determined from cholesteryl ester. The fat value has been used, principally because of the similarity of its ester type to that of lecithin. The value of cholesterol at this wave length turns out to be small, whether evaluated from cholesteryl ester or from unesterified cholesterol. Tentatively, it has been assigned a value of 0, resulting in a further simplification of the calculations. The equations are now reduced to the following:

$$A^{5.8} = 0.10 \times C_{EFA}$$

$$A^{7.25} = (0.018 \times C_{EFA}) + (0.0274 \times C_{TC}) + (0.018 \times C_{RP})$$

$$A^{9.15} = (0.023 \times C_{EFA}) + (0.125 \times C_{RP})$$

The calculation can be performed by first determining esterified fatty acids from the 5.8 μ measurement. Then corrections are determined for this component at the 7.25 μ and 9.15 μ bands. At 9.15 μ (1090 cm.$^{-1}$) only one term remains, and the C_{RP} determined can be converted to total phosphatides by a factor that assumes an approximately constant percentage of fatty acids. A second correction is made for phosphatides at 7.25 μ, and the net absorbance is used to determine total cholesterol.

Since there are numerous assumptions and averages involved in this development, there is a limit to the range of lipide compositions for which the analysis is appropriate, aside from considerations of accuracy of measurement. Whether the range encountered in serum lipides can exceed that limit remains to be seen. Five serum samples chosen at random have been analyzed in this way,

TABLE 3
INFRARED ANALYSES OF LIPIDE EXTRACTS FROM SERA (RANDOM SAMPLES): COMPARISON WITH CHEMICAL DATA

	Milligrams per milliliter				
	1	2	3	4	5
Esterified fatty acids (5.8 μ)	5.29	5.24	4.15	4.75	5.68
Phosphatides (9.15 μ)	2.29	2.44	2.18	1.82	2.78
Total cholesterol (7.25 μ)	2.10	2.76	2.30	1.80	3.07
Total fatty acids*	5.48	5.00	3.94	4.35	5.28
Phosphatides (chemical)	2.64	2.72	2.15	1.88	2.82
Total cholesterol (chemical)	1.99	2.65	2.68	1.95	3.15
Total lipide (6.8 μ)	8.85	9.38	7.66	7.66	10.40

* By saponification and infrared measurement of carboxyl.

with the results shown in TABLE 3. A total lipide value has been included, determined from the 1470 cm.$^{-1}$ (6.8 μ) band intensity and an empirical average absorptivity for the various components. As a first and only trial the results, in comparison with chemically determined values, are very encouraging. Needless to say, much more testing and statistical evaluation must be done to learn the limitations of this method. If its validity can be established to a satisfactory degree, it should be possible easily to reduce the amount of serum required to a few tenths of a milliliter, and probably even to less with appropriate handling techniques.

Fatty Acid Unsaturation

Infrared spectrometric methods for measuring the total number of *cis*, unconjugated double bonds in fatty acids have been proposed by Sinclair *et al.*[13] One method was based on the olefinic carbon-hydrogen (C=C H) stretching vibration band at 3040 cm.$^{-1}$ (3.3 μ). A function of this band and the aliphatic C H absorption intensity at 2920 cm.$^{-1}$ (3.42 μ) could be related linearly with the number of *cis* double bonds per molecule. In our laboratory we have shown a similar relationship between the 3040 cm.$^{-1}$ band intensity and the iodine numbers of olive, cottonseed, corn, and linseed oils. Perhaps the reason this method has not been exploited more is that the band is usually not well resolved when a sodium chloride prism is used. Most instruments in general use are not equipped with the required lithium fluoride or calcium fluoride prism.

A second absorption band of *cis* double bonds that might be employed is a rather broad and diffuse one in the 14 μ region. Sinclair *et al.*[13] observed that on cooling the unsaturated fatty acids to -196° C. the character of this band changed, with the appearance of sharp bands of differing patterns for oleic and linoleic acids. It was suggested that binary mixtures of those two acids could be analyzed on some empirical basis from the low-temperature solid-phase spectra. This method suffers from complexities of the experimental technique.

Brominated fatty acids were distinguishable spectroscopically, but chiefly in the wave-length range beyond 15 μ. This range is not accessible with a sodium chloride prism, but could be reached with potassium bromide.

A possible approach to this problem that we have considered is to measure the integrated intensity of the 14 μ band over some arbitrary interval of wave length and to try to correlate this quantity with total unsaturation. Judging from the broadness and relatively low intensity (maximum) of this absorption, it seems that integration should provide a better measure than a single measurement of absorbance. At present we have no data bearing on this point.

Potential Clinical Laboratory Applications of Infrared Spectroscopy

Infrared methods applied to medical problems have so far been largely within the orbit of research. It is natural that they should begin there; and the techniques are, after all, relatively new, having been developed during the last ten years. The research phase is necessary, not only to develop experimental procedures and build up the background for interpretation of spectral information, but to find specific applications or to establish the areas in which they are likely to emerge. The time may well be approaching when the next phase of the evolution toward broader usage can be entered. From the experimental standpoint, this may involve simplification of procedures, automatic operation and analysis and, perhaps, the use of nondispersive analyzers.

The other major factor bearing on this development is the medical requirement for analyses for which infrared methods are well suited. Consider serum lipides as an example. In clinical routine, blood samples are certainly among the most common sources of diagnostic information. For the lipides of blood serum it is pertinent to inquire: first, what lipide determinations have diagnostic value; and, second, whether any of these determinations can be made advantageously by infrared methods with respect to reliability, time, or effort. The measurement of cholesterol may be placed in the category of a diagnostic test because of its statistical correlation with the risk of coronary heart disease. Evidence has been put forth by Nichols, Lindgren, and Gofman[14] at our laboratory that the concentration of total lipides determined gravimetrically has an even better correlation with risk of coronary disease than does cholesterol (using lipoprotein levels as a basis of reference). This suggests a possible demand for a simple and reliable analysis of total lipides in small amounts of serum. While infrared measurement of cholesterol, as we have performed it, is less accurate than established chemical methods, it may well be sufficiently accurate for clinical use. On the other hand, it has been indicated in a preceding section of this paper that infrared spectrophotometry is potentially capable of yielding estimates, not only of cholesterol, but of total lipides, total esterified fatty acids, and total phosphatides, and that all of these may be determined in the same analysis by a procedure that is simpler than the chemical determination of cholesterol alone. Whether or not this method is proven worthy by more extensive testing, it is an example of one type of simplification that might conceivably lead to more widespread use of infrared methods in clinical medicine.

Acknowledgment

A large part of the work on chromatography of phosphatides has been performed by Gary Nelson.

References

1. Potts, W. J., Jr., & N. Wright. 1956. Anal. Chem. **28:** 1255.
2. Blout, E. R. & R. C. Mellors. 1949. Science. **110:** 137.
3. Woernley, D. L. 1952. Cancer Research. **12:** 516.
4. Gofman, J. W., O. deLalla, F. Glazier, N. K. Freeman, F. T. Lindgren, A. V. Nichols, B. Strisower & A. R. Tamplin. 1954. Plasma. **2:** 413.
5. Freeman, N. K., F. T. Lindgren, Y. C. Ng & A. V. Nichols. 1953. J. Biol. Chem. **203:** 293.
6. Borgström, B. 1952. Acta Physiol. Scand. **25:** 101, 111.
7. Freeman, N. K., F. T. Lindgren, Y. C. Ng & A. V. Nichols. 1957. J. Biol. Chem. In press.
8. Lindgren, F. T., A. V. Nichols & N. K. Freeman. 1955. J. Phys. Chem. **59:** 930.
9. Lea, C. H., D. N. Rhodes & R. D. Stoll. 1955. Biochem. J. **60:** 353.
10. Marinetti, G. & E. Stotz. 1954. J. Am. Chem. Soc. **76:** 1347.
11. Renkonen, K. O. & R. Koulumies. 1953. Ann. Med. Exptl. et Biol. Fenniae Helsinki. **31:** 248.
12. Sperry, W. M. & F. C. Brand. 1955. J. Biol. Chem. **213:** 69.
13. Sinclair, R. G., A. F. McKay, G. S. Myers & R. N. Jones. 1952. J. Am. Chem. Soc. **74:** 2578.
14. Nichols, A. V., F. T. Lindgren & J. W. Gofman. 1957. Geriatrics. **12:** 130.

THE CHARACTERIZATION OF MYCOBACTERIAL STRAINS BY THE COMPOSITION OF THEIR LIPIDE EXTRACTS*

By D. W. Smith†, H. M. Randall‡, M. M. Gastambide-Odier†, and A. L. Koevoet‡

Department of Medical Microbiology, University of Wisconsin, Madison, Wis.
and
Harrison M. Randall Laboratory of Physics, University of Michigan, Ann Arbor, Mich.

After a brief introduction, we shall present studies on the reproducibility of the techniques, a consideration of the sensitivity of the method and, finally, an application that is presently under consideration.

In 1948 a research program studying the immunizing properties of fractions of the tubercle bacillus was in progress in the Bacteriology Department of the University of Michigan. In this study, complex extracts were prepared from mass cultures of the organism and were tested in guinea pigs and mice for their capacity to cause alterations in the normal resistance of the animals to tuberculosis.

About this time, one of us (HMR) approached the Bacteriology Department with an inquiry as to whether there were any lines of research in which infrared spectroscopic studies could make a contribution to the study of complex biological mixtures. The feasibility of such an investigation in the work on immunity in tuberculosis was evident. The fact that the extracts being prepared from the tubercle bacillus were of a lipide nature proved to be an important factor in the progress made in the present research.

Studies on Reproducibility

After several abortive attempts to find a correlation between the immunizing potency of a given preparation and its spectrum, we decided that it would first be necessary to study the system with which we were working to determine the degree of reproducibility of bacterial extracts prepared under similar conditions and the extent to which the different variable factors could be altered without affecting the extracts.

Working with total $CHCl_3$-soluble fractions and subfractions prepared by precipitation with acetone, we were able to show[1] that, with sufficient care, different lots of tubercle bacilli grown and extracted under similar conditions yielded substances, the infrared spectra of which were sufficiently reproducible to warrant further study. Following this, a study was made of the effects of age of the inoculum, age of culture, temperature of incubation, composition of the medium, and duration of extraction on the reproducibility of the infrared spectra of the lipide extracts. It was determined that all of these factors were critical and that, if varied, they would lead to changes in the composition of the lipide mixtures.

* This work was supported by grants from the Research Corporation, New York, N. Y., the Wisconsin Anti-Tuberculosis Association, Milwaukee, Wis., and the Rackham Fund of the Graduate School, University of Michigan, Ann Arbor, Mich.

† University of Wisconsin.
‡ University of Michigan.

Sensitivity

Having chosen a set of conditions that would lead to spectroscopically reproducible lipide extracts, it was decided, as a measure of the sensitivity of the method, to determine whether it could distinguish between strains known to be biologically distinct. The following groups of organisms were chosen so that each succeeding study involved a more subtle biological difference and, possibly, a more subtle chemical difference.

(1) The first study was concerned with two strains, H37Rv and H37Ra, derived from a single parent culture, one being virulent and the other avirulent. The cultures can be distinguished grossly.

(2) As a second phase of the study, human, bovine, and avian varieties of the tubercle bacillus were examined. The human and bovine varieties are very similar, differing only in the fact that bovine bacilli produce progressive disease in rabbits while human bacilli do not. Both produce progressive tuberculosis in guinea pigs and in man.

(3) Next to be examined were recently isolated strains of human tubercle bacilli. We were looking for chemically distinct subgroups, since there are no recognized biological subgroups of the human variety of bacillus.

(4) The last phase of the work that will be discussed, the one now under investigation, is concerned with a study of differences in lipide composition between strains of mycobacteria isolated from human sputum; some of these, namely tubercle bacilli, are capable of producing disease in guinea pigs, while others do not have this property. The latter strains are called atypical acid-fast bacilli.

Throughout the course of this study improvements have been made in the manner of recording spectra, in the methods of preparing extracts, and in their subsequent resolution into simpler mixtures and single compounds. For this reason, details of the methods of separation used in the early studies will not be presented here.

For the study of H37Rv and H37Ra,[2] identical procedures were carried out on the two strains. Mass cultures of both strains were prepared on a synthetic medium, the cells were harvested at six weeks, and extracted with a mixture of equal parts of methanol and $CHCl_3$. This extract was filtered to remove the cells and then was cooled to 5° C. The white solid that precipitated was removed and dried at the same temperature. The infrared spectra of the 5° C. insoluble fractions from both strains are shown for comparison in FIGURE 1. The differences have been shown to be reproducible, and they indicate chemical differences in the two strains, either qualitative or quantitative, or both.

Turning next to a study of possible differences in lipide composition between human and bovine tubercle bacilli,[3] mass cultures were prepared and extracted under conditions similar to those mentioned in the work on the H37 strains. This time the methanol-chloroform extract was precipitated at 5° and the filtrate was again precipitated at −30° C. In FIGURE 2 are shown for comparison the infrared spectra of whole cells of human and bovine bacilli (upper two spectra) and the total lipide extracts (lower two spectra). Although minor spectral differences exist, they are not sufficiently reproducible to be of help in distinguishing the strains. In FIGURE 3 are shown the spectra of the 5° and

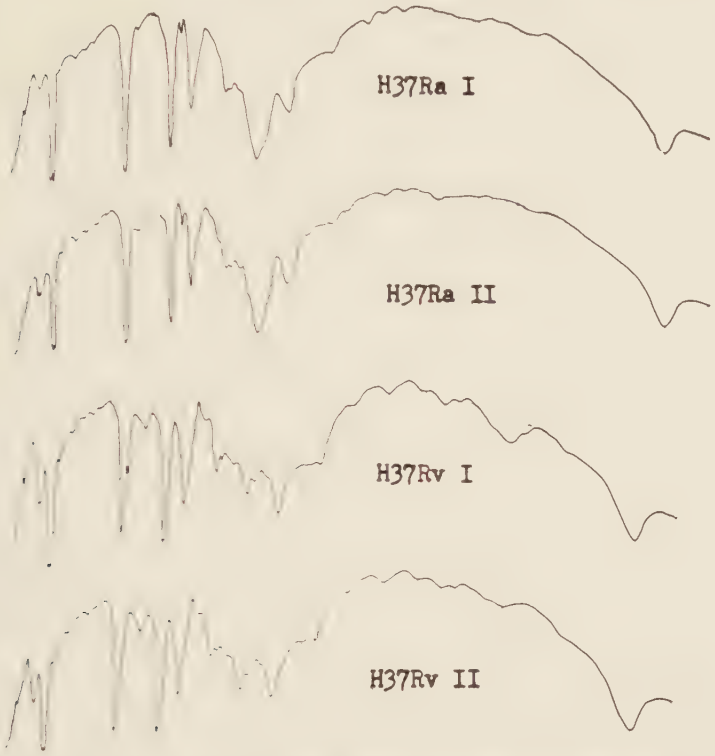


FIGURE 1. Infrared spectra of the 5° C. insoluble fractions of H37Rv and H37Ra. Lot I of each strain was harvested in March of 1951, lot II in May of 1951.

−30° C. insoluble fractions for a human and bovine strain. The 5° fractions have proved to be very reproducible, but no significant spectral differences can be seen. The −30° C. fraction lacks the same degree of reproducibility, and it was only after a large number of these fractions had been prepared and spectra recorded that the minor band at 1510 cm.$^{-1}$ (6.63 μ) was observed to be uniformly present in the fractions from bovine strains and not in the corresponding fraction from human strains.

Substantial masses of the −30° C. insoluble fraction of two bovine and one human strain were prepared and subjected to further separation by batch adsorption chromatography on Magnesol*. The fractions eluted from the adsorbent in ether are shown in FIGURE 4. It can be seen that a considerable concentration of the compound responsible for the 1510 cm.$^{-1}$ band has occurred in the case of the lipides of the two bovine strains, while none of this material can be detected in the fractions of the human-strain lipides. Subsequently this substance was demonstrated to be a single compound present in the lipides of seven bovine strains and absent in the lipides of twenty human strains.

The next phase of this study was concerned with an attempt to detect

* Magnesol—magnesium silicate, Westvaco Chemical Co., Chlor Alkali Div., South Charleston, West Va.

FIGURE 2. Infrared spectra of the whole cells (Nujol mull) and total lipides of human and bovine tubercle bacilli.

chemical differences between strains of human tubercle bacilli recently isolated from the sputum of patients.[4] At this time we began employing column chromatography to achieve better separations. Cultures were extracted with a mixture of equal parts of ethanol and diethyl ether, and the extract was partitioned between ether and water to remove the glycerol. Twenty cultures have been prepared and extracted in this manner and the extracts chromatographed on Magnesol-Celite*. For the most part the results indicate a similarity of lipide composition. In only one strain was a compound found to be absent in the ether-soluble lipides when it was known to represent 15 per cent of the lipides of all other human strains. Even in this one strain, the missing compound could be demonstrated in the lipides later removed from the strain in $CHCl_3$.

Examples of the types of substances found in the human tubercle bacillus are shown in FIGURES 5 and 6. Because of the rather uniform distribution in the

* Celite—Analytical Filter Aid, Johns Manville Co., New York, N. Y.

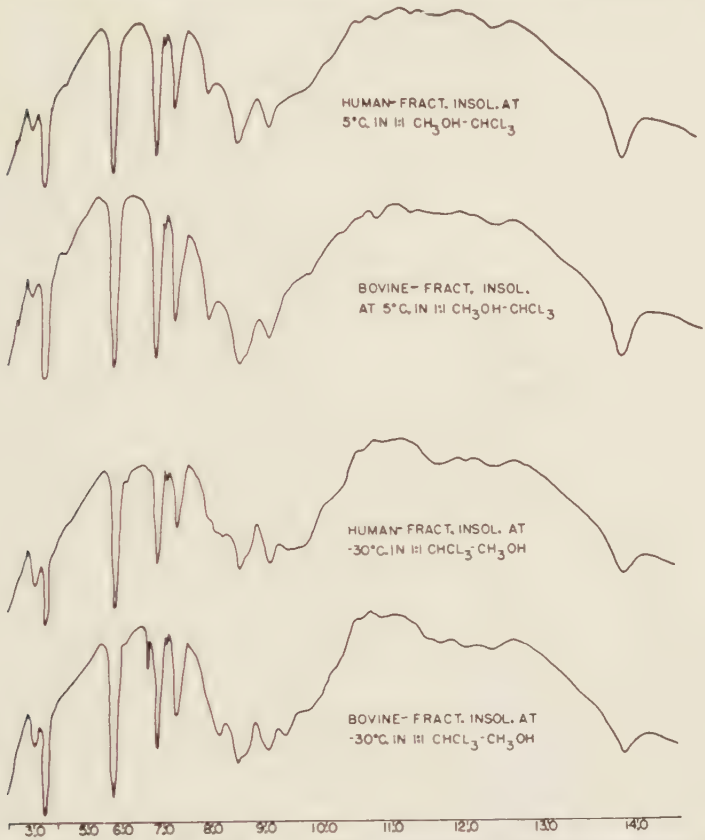


FIGURE 3. Spectra of the 5° C. and −30° C. insoluble fractions prepared from the lipides of human and bovine tubercle bacilli.

various types of mycobacteria of the series of compounds shown in FIGURE 5, only limited chemical studies have been made. On the basis of chromatography, of saponification products, and of comparison of the spectra with spectra of known materials, compound A would appear to be a triglyceride, compound C a diglyceride, and compound D a monoglyceride. The spectrum of the fatty acid shown is only a representative of the group of fatty acids present in lipides of mycobacteria. This group is resolved, by chromatography on rubber columns, into the following: palmitic, stearic, oleic, and tuberculostearic acids, and others only partially separated.

The compounds shown in FIGURE 6 are not as uniformly distributed in all of the human strains. Mycolic acid, empirical formula $C_{87}H_{174}O_4$, and dimycocerenate of phthiocerol, empirical formula $C_{93}H_{188}O_5$, were identified by comparison of their spectra with spectra of the pure compounds isolated from mycobacteria by Asselineau and Lederer.[9] Cord factor, a toxic glycolipide, empirical formula $C_{186}H_{366}O_{17}$, was identified by comparison of spectra and melting points with the pure compound isolated by Noll.[6]

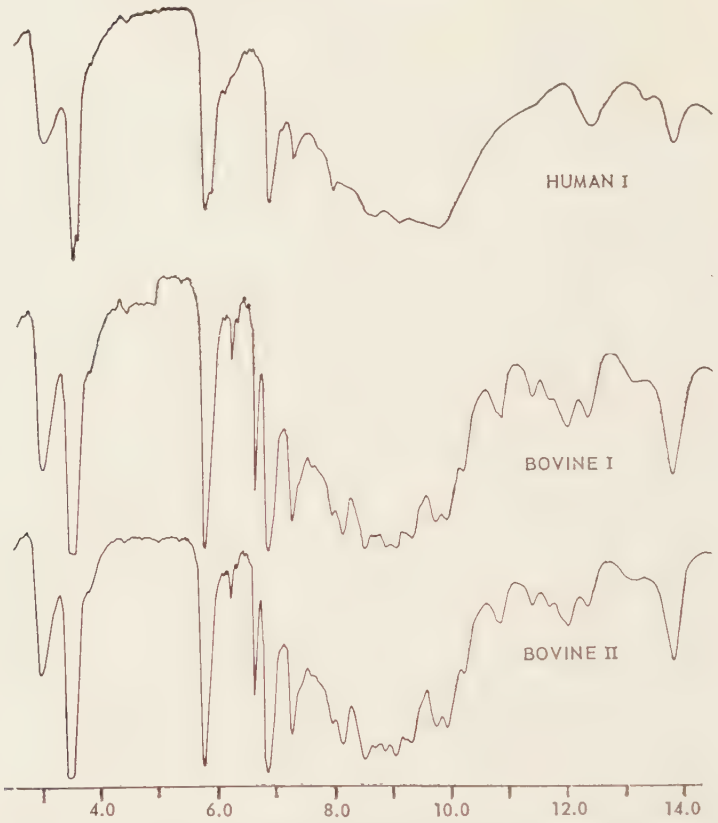


FIGURE 4. Spectra of the ether eluates separated by batch adsorption from the −30° C. insoluble fractions of two bovine cultures and one human culture.

We should like to give primary emphasis to the work now in progress, in which we are studying differences in lipide composition between human tubercle bacilli and the so-called atypical acid-fast bacilli. These organisms are similar to tubercle bacilli in many respects: they are acid-fast, and are repeatedly isolated from sputum or from surgical-resection material from patients ill with a disease similar in all respects to tuberculosis. However, they do not produce progressive disease in guinea pigs—a criterion that previously had been used to decide whether or not a given strain was tubercle bacillus or not. There are as yet no recognized biological tests for the atypical strains.

A series of seven atypical cultures was obtained from recognized sources and, at the time of receipt, all the cultures were said to fall into the atypical classification. These strains, together with other known varieties of acid-fast bacilli (BCG, *Mycobacterium phlei*, avian, bovine, and human strains), were cultured on a synthetic medium for six weeks at 37° C. The cultures were harvested, and the medium was withdrawn, autoclaved, and discarded. The cell masses of 25 one-gallon jug cultures of each strain were combined in a mixture of equal

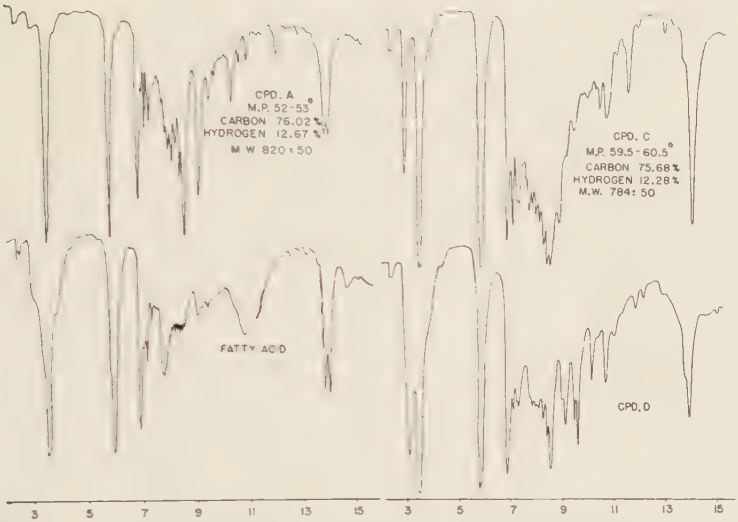


FIGURE 5. Infrared spectra of four substances found to be present in various types of mycobacteria.

parts ethanol and ether and were extracted for 48 hours. A second 24-hr. extraction with fresh ethanol-ether followed. The cells were separated from the solvent by filtration, were allowed to dry, and then were extracted for 24 hours with $CHCl_3$. The ethanol-ether extract was concentrated to a minimum volume and then partitioned between water and ether. The ether-soluble fraction was dried over magnesium sulfate, the ether was removed under vacuum, and the dry lipide was stored at 5° C. until it was chromatographed. The flow diagram for these operations is shown in CHART 1.

Chromatography was carried out in 50 mm.-diameter columns packed with 85 gm. of a mixture of equal parts Magnesol and Celite that had been washed with acetic acid, hot distilled water, ether, and methanol, and then activated at 150° C. for 30 min. The lipide material was applied to the column in hexane, the solvent used to make the slurry with which the column was packed. The columns were eluted sequentially with a series of solvents of increasing polarity, beginning with washed redistilled hexane and ending with mixtures of acetic acid in ether. Columns were treated as flowing chromatograms, and the eluates, 250 cc. each, were collected as they flowed from the column. The quantity of each solvent used varied according to the amount of material found in the eluates. The eluates were evaporated to dryness and weighed, and the infrared spectra were recorded on a Model 21 Perkin-Elmer double-beam spectrophotometer.

TABLE 1 gives the main compounds found in the different strains studied. The fatty acids and esters of fatty acids common to all strains are not included. Spectra of some of these substances are to be found in FIGURES 5 and 6. Free mycolic acid or its analogues and cord factor are found in most, but not all, of the different types of bacilli.

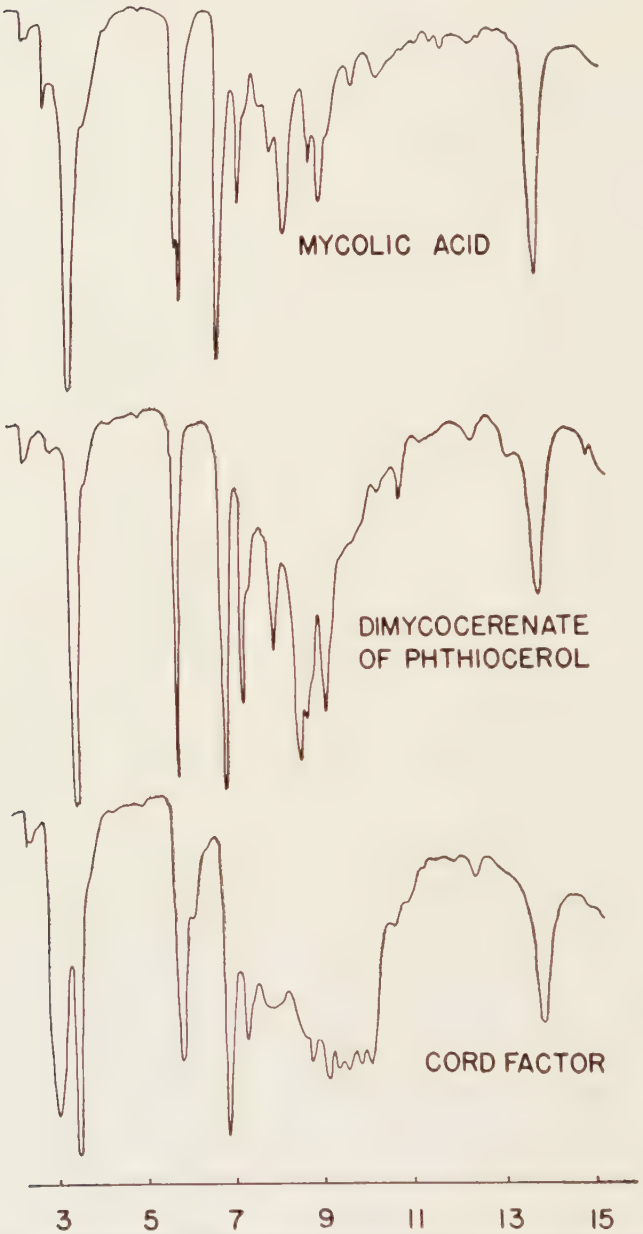


FIGURE 6. Infrared spectra of three compounds identified in the lipides of some cultures of the human variety of tubercle bacillus.

CHART 1

FLOW DIAGRAM FOR THE EXTRACTION OF LIPIDES OF THE TUBERCLE BACILLUS

Cultures grown 6 weeks at 37° C. (modified Long medium)

Media removed under vacuum; cells allowed to stand in 1:1 ethanol-ether for 48 hours, then collected on Buchner funnel

Original ether-ethanol extract evaporate to dryness

Cell mass

Re-extract with ethanol-ether for 24 hours

Extract

Concentrate to dryness, pool with original, and partition between water and ether; chromatograph the ether-soluble fraction

Cell residue

Extract with $CHCl_3$ (24 hours)

Extract

Concentrate to dryness and prepare for chromatography

Cell residue

Discard

TABLE 1

LIPIDE SUBSTANCES ISOLATED FROM VARIOUS STRAINS OF MYCOBACTERIA

Compounds	Distribution in the various strains									
	Atypical*			Human†	Bovine		Avian		Saprophyte‡	
	I	II	III		Vir.	BCG	I	II	10-day	28-day
Compound Ga	+				+	+				
Compound Gb			+				+	+		
Compound J										
Dimycocerenate of phthiocerol	+			+	+					+
Cord factor		+		4/5	+		+		+	+
Mycolic acids				6/20						

* Four strains in group I, two in group II, and one in group III.

† Twenty virulent human strains.

‡ *M. phlei* harvested one time after 10 days' incubation and a second time after 28 days' incubation.

Since cord factor and mycolic acid are so widely distributed in the various strains, they cannot be used generally as a means of differentiation. There are among the isolated substances three compounds of particular significance in classifying these strains: dimycocerenate of phthiocerol (DIM) and two unidentified compounds referred to as Ga and Gb. DIM, shown in FIGURE 6, has thus far been found only in human and bovine strains and represents approximately 15 per cent of the total lipides. Compound Gb has been found only in bovine strains and is the compound referred to earlier in discussing bovine tubercle bacilli. The isolation of a spectroscopically identical compound from a bovine strain was confirmed by Noll.[7] Compound Ga has been found repeatedly in the lipides of some of the atypical strains. Two of these strains, obtained from different sources and differing somewhat in growth characteristics, have been cultured 3 times each, and the extracts have been chromato-

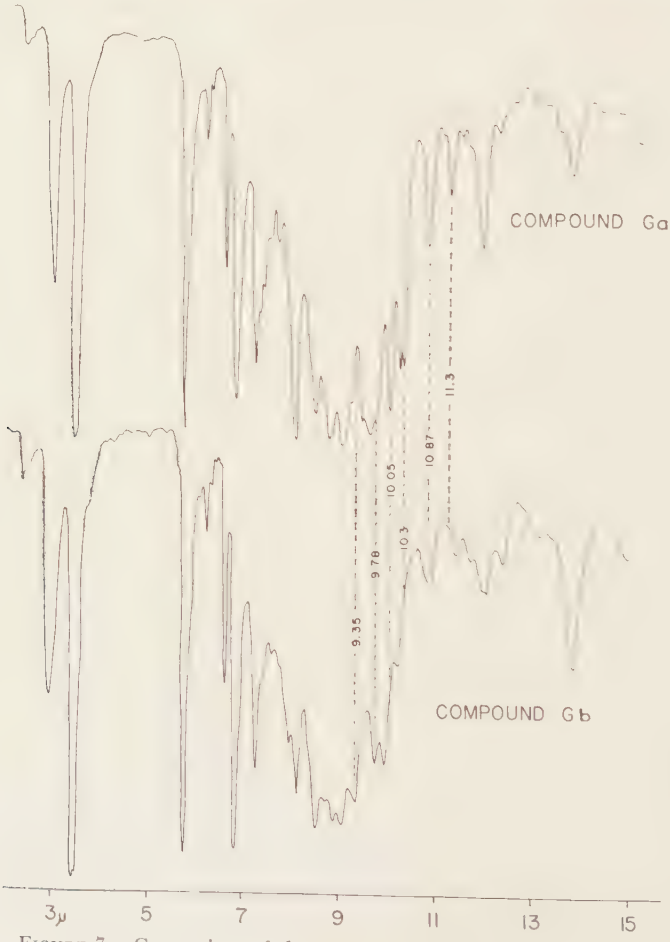


FIGURE 7. Comparison of the spectra of compounds Ga and Gb.

graphed from 5 to 8 times with the demonstration of compound Ga on each occasion. Compound Ga could not be detected in the lipides of 3 of the 7 strains originally labeled atypical. Concerning 2 of these cultures lacking this substance (see TABLE 1, atypical group II), after this study was completed, it was suggested by the investigator from whom the cultures were received that, since these cultures were isolated frequently in routine cultures and have never been identified in the tissues, they should probably be considered saprophytes. Our findings are in agreement with this suggestion. The third strain of the atypical group, lacking Ga, has been found to contain a different substance, designated J, which otherwise has been found only in the 2 avian strains. Additional studies will be required to establish the significance of J.

Thus far it would appear that we have isolated two substances, Ga and Gb, the spectra of which are shown for comparison in FIGURE 7, which may be specific for the types of bacilli from which they were isolated. This possibility is made even more interesting by the fact that the infrared spectra of the two substances indicate a rather close chemical relationship. It is possible that the study of other atypical strains will lead to the recognition of other types and that each subgroup might be identified by a compound or group of compounds.

In TABLE 2 we have summarized the present information about Ga and Gb. Both are fairly resistant to saponification, being split completely only after 72 hr. at 80° C. In each case, the saponification products are an acid and an alcohol.

If the data that we have obtained thus far are confirmed, it would appear that it is possible to distinguish between each of the major subgroups of mycobacteria examined. The different varieties of mycobacteria could be grouped first on the basis of the presence or absence of DIM. Human and bovine strains, both containing DIM, could be separated by the presence of Gb in bovine strains only. The subgroups lacking DIM include atypicals, BCG, avian strains, and saprophytes. The atypical strains could be recognized by the presence of Ga, BCG strains by the presence of Gb, avian strains by the presence of J, while the saprophyte strains contain none of the specific compounds. These observations are summarized in TABLE 1.

TABLE 2

COMPARISON OF THE CHEMICAL AND PHYSICAL PROPERTIES OF COMPOUNDS GA, GB AND THEIR ALCOHOLIC DERIVATIVES

	Compound			
	Ga	G'a	Gb	G'b
Melting point	—	—	—	40° C.
Carbon (%)	72.5	64.2	77.1	67.2
Hydrogen (%)	11.2	9.77	12.1	10.8
Nitrogen	absent	absent	absent	absent
Mol. weight	1000	830	1000	800
U.V. maxima	275, 282	275, 282	275, 282	275, 282
Type of compound	ester	alcohol	ester	alcohol

Discussion

As far as the present work is concerned, we feel that the next step is to extend our findings to include a greater number of atypical strains. We should then like to develop a simple means of isolating Ga, or its equivalents if subgroups of atypicals exist, so that its presence or absence in a given strain could be determined quickly. Ultimately, if this test is to be a practical means of determining whether a strain is atypical or not, it should be developed in a manner to eliminate the time-consuming expensive steps, including mass-culture preparation, chromatography, and spectroscopy. It might be possible to take advantage of specific chemical groups of Compound Ga in devising a color development test that could be performed on the patient's primary sputum culture.

It would be of interest to determine whether the preliminary results on classification of the various other groups of mycobacteria are still valid after more strains have been examined. Since the mycobacteria are not as well classified by immunological means as other groups of bacteria, a grouping based on lipide content might serve a useful purpose. Moreover, where a strain-specific substance, such as Gb, is isolated, it would be interesting to determine whether the substance plays any role in the biological differences observed among the varieties of tubercle bacilli involved.

Thus far, this research has been entirely on a qualitative basis; that is, a compound is either present or not present. Quantitative studies might show significant differences between strains on the basis of relative amounts of some of the substances now found to be present in all strains.

We have made only very limited studies of bacteria outside the genus *Mycobacterium*. We compared the spectra of the whole cell and of a total lipide extract for each of eighteen types of bacteria* and found considerable specificity to the spectrum of the extract and less specificity to the spectrum of the whole-cell preparations. We see little hope for the use of infrared spectroscopy as a practical means of identifying bacteria in general, particularly when the method must compete with the highly specific techniques involving the use of fluorescent antibody.

Speaking in more general terms, we feel that we have demonstrated the utility of infrared spectroscopy as applied to the detection of differences in lipide composition among complex biological materials. Although the mycobacteria have been extremely valuable for study because of their high lipide content, it is possible that the techniques employed in this research could be useful in comparing, for example, normal and diseased tissues.

Acknowledgment

We acknowledge our indebtedness to Paul Maker, Richard Putney, and Harold Patterson for their valuable assistance in this work.

* Including members of the following genera: *Neisseria*, *Bacillus*, *Escherichia*, *Proteus*, *Staphylococcus*, *Aerobacter*, *Klebsiella*, *Micrococcus*, and *Pseudomonas*.

Addendum

Since this paper was submitted for publication, additional atypical strains have been studied, bringing the total now to 13 strains. All of the new strains fit into the chemical classification developed in the earlier studies.

In addition, progress has been made on the chemical structure of the strain-specific compounds Ga, Gb, and J. Ga and Gb appear to be closely related to the dimycocerenate of phthiocerol (DIM). The main points of difference are that the G compounds contain only one mycocerosic acid unit and the methoxyl group of DIM is absent in the G compounds, which contain instead a substituted aromatic ether. The G compounds also contain a free OH group. Compound J has now been resolved into two closely related but spectroscopically distinct compounds, one of which is characteristic for avian tubercle bacilli and the other characteristic for the group III atypical strains.

References

1. Randall, H. M., D. W. Smith, A. Colm & W. J. Nungester. 1951. Correlation of biologic properties of strains of *Mycobacterium* with their infrared spectrums. I. Reproducibility of extracts of *M. tuberculosis* as determined by infrared spectroscopy. Am. Rev. Tuberc. **63:** 372.
2. Randall, H. M., D. W. Smith & W. J. Nungester. 1952. Correlation of biologic properties of strains of *Mycobacterium* with their infrared spectrums. II. The differentiation of two strains, H37Rv and H37Ra of *M. tuberculosis* by means of their infrared spectrums. Am. Rev. Tuberc. **65:** 477.
3. Smith, D. W., W. K. Harrell & H. M. Randall. 1954. Correlation of biologic properties of strains of *Mycobacterium* with their infrared spectrums. III. The differentiation of bovine and human varieties of *M. tuberculosis* by means of infrared spectrums. Am. Rev. Tuberc. **69:** 505.
4. Kubica, G. P., H. M. Randall & D. W. Smith. 1956. Correlation of biologic properties of strains of *Mycobacterium* with their infrared spectrums. IV. Fractionation and comparison of the lipids of human strains of *Mycobacterium tuberculosis* by means of their infrared spectrums. Am. Rev. Tuberc. Pulmonary Diseases. **73:** 529.
5. Asselineau, J. & E. Lederer. 1953. Chimie des lipides bactériens. Progr. chim. substances org. nat. **10:** 170.
6. Noll, H. 1956. The chemistry of cord factor, a toxic glycolipid of *M. tuberculosis*. Fortschr. Tuberkulose Forsch. **7:** 149.
7. Noll, H. 1957. The chemistry of some native constituents of the purified wax of *Mycobacterium tuberculosis*. J. Biol. Chem. **224:** 149.

THE STUDY OF VIRUS PREPARATIONS BY INFRARED SPECTROSCOPY*

By Albert A. Benedict†

Department of Preventive Medicine and Public Health, University of Texas Medical Branch, Galveston, Texas

Infrared spectroscopy has been mainly used by the chemist and physicist as a means for identifying and determining the molecular structure of low-molecular-weight substances. In spite of limitations imposed by instrumentation, biologists have attempted to apply the technique for a variety of purposes. The classification of heterogeneous mixtures of large molecules is one of the most impressive challenges to the inherently low "signal-to-noise" ratios induced by large molecules. Minor molecular alterations, such as the addition or subtraction of carboxyl or methyl groups, are not detectable in specimens of unfractionated tissues and microorganisms. Nevertheless, there is considerable evidence to indicate that biological systems can be studied by infrared spectroscopy,[1-6] and that in certain instances the method can be used for the identification of constituents in mixtures of macromolecules.[7-10] The unique spectra obtained with specimens of whole bacteria[2] and with virus preparations, for example, can be attributed in most cases to quantitative differences among the major biochemical components rather than to qualitative differences within similar molecular structures. In view of this, the reproducibility of the unique features of the spectra of such materials remains the major basis for taxonomic differentiation. Thus, precise conditions for the preparation of tissues or microorganisms are required to fulfill this criterion.

The criteria available for taxonomic arrangement of viruses depend upon host and tissue preference, antigenic structure, hemagglutination phenomena, size, morphological characteristics, and susceptibility to inactivation by physical and chemical agents. In addition, investigations concerned with the biochemical constitution of viruses may lead to a rational method of distinguishing these agents. In view of the doubtful purity of most virus preparations, at this time, only questionable biochemical data are available for classification purposes.

Nevertheless, the application of infrared absorption spectroscopy was suggested.[11] The initial studies[11] failed to show the presence of sharp absorption bands, and, in view of the biochemical make-up of viruses, these reported spectra were surprising. A spectrum of a Newcastle disease virus (NDV) preparation showed only minimal absorption in the vibrational region of 1500 to 1700 cm. $^{-1}$, suggesting that perhaps amide groups were present, but that the quantities of material analyzed were insufficient to yield major and minor bands. Apparently no effort was made to determine the concentrations required to produce distinctive bands.

* The study on which this paper is based was supported by funds provided under Contract AF 18(600)-464 with the United States Air Force School of Aviation Medicine, Randolph Field, Texas.

† Present address: Department of Bacteriology, University of Kansas, Lawrence, Kans.

Following this attempt to differentiate viruses, we endeavored[12] to obtain fractions of infected chick embryo allantoic fluid (AF) that could best serve for identification purposes. Purity of the virus preparations was not seriously considered, since the criterion used for identification was based on reproducibility of spectral characteristics. In order to maintain a spectrally constant environment, all of the viruses studied were adapted to propagate in the developing chick embryo by allantoic cavity inoculation, and all of the allantoic-fluid viral suspensions were harvested from 12-day-old embryos. These fluids were cleared by slow-speed centrifugation, and the supernates (crude AF) were used for purification purposes. Fluids from normal 12-day-old embryos were also processed in the same fashion.

The magnitude of variation in the spectra either of crude fluids from single eggs infected with the same virus or of fluids from single normal eggs was so great that it was impossible to assign identifying characteristics. The variation was particularly evident between 1000 to 1200 cm.$^{-1}$ and 825 to 900 cm.$^{-1}$ In the case of fluids infected with the influenza strains, with Newcastle disease virus (NDV), or with mumps, there was no correlation between the spectral variations and the viral content as measured by hemagglutinin activity. However, when the spectra of different pooled infected fluids were compared to the spectrum of normal allantoic fluid, the appearance of exaggerated bands at about 1040 to 1125 cm.$^{-1}$ (8.9 to 9.6 μ) was sufficiently consistent at least to indicate virus infection (FIGURE 1). Supernates of the pooled AF obtained by centrifugation of the fluid at 100,000 $\times$ g for 45 minutes yielded the same spectra as did the uncentrifuged fluid. Centrifugations at this speed removed most of the infective particles and hemagglutinin, and thus the variations observed probably represented host metabolic products and, or soluble viral products.

The system[11] previously mentioned was reinvestigated. The method used consisted of sedimentation of the viruses from cleared AF by a single high-speed centrifugation, and resuspension of the viral pellets in water to one tenth of the original AF volume. The spectra produced by these preparations showed that the concentrations used were insufficient to give distinct bands. Further concentration of such pellets yielded spectra composed of sharp bands (FIGURE 2). As with crude AF, these spectra were not reproducible, although infected material was distinguishable from normal host components (FIGURE 2). The absorption bands at 1650, 1540, 1450, 1230, 1150, and 1060 cm.$^{-1}$ (6.05, 6.5, 6.9, 8.1, 8.7, and 9.4 μ) were resolved in preparations of influenza A having high hemagglutinin titers. As will be shown later, the spectra of the normal host components (FIGURE 4) were altered by further purification, eventually acquiring the characteristics of the virus preparations. The most striking effect was the disappearance of the substances responsible for absorption at about 1590 and 1410 cm.$^{-1}$ (6.3 and 7.1 μ).

Thus, the use of crude fractions was complicated by the inherent variation in the ratios of contaminating host materials to virus content. The marked variation of the spectra of crude AF observed among individual normal embryos suggested that the viruses were not propagated in a spectrally homogeneous medium. As with the crude normal AF, the spectra of different individual

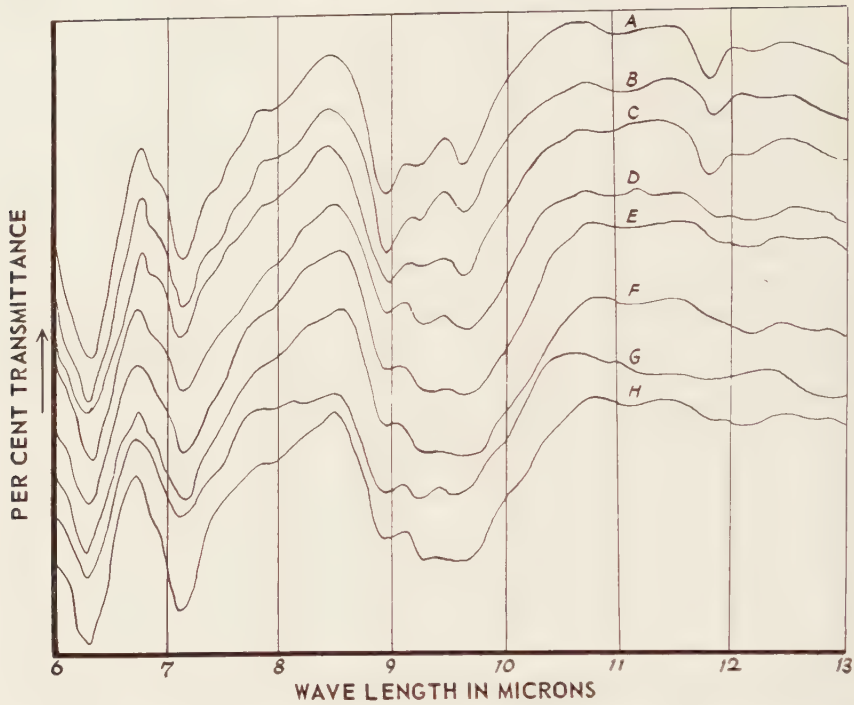


FIGURE 1. Spectra of virus-infected allantoic fluids: A, psittacosis; B, meningopneumonitis; C, influenza A'; D, influenza B; E, influenza A; F, mumps; G, Newcastle disease; H, normal.

eggs with the same virus infection were not the same. Some "ironing out" of these variations resulted from pooling of normal or of infected fluids.

Partial purification of the viruses by three cycles of differential ultracentrifugation gave qualitatively reproducible absorption spectra,[13] thereby making possible the detection of subtle differences among some of the different viruses. FIGURE 3 shows the reproducibility of four separate preparations of meningopneumonitis virus (MP). FIGURE 4 presents partial spectra of the viruses studied to date, and of the normal host preparation. Four groups were established on the basis of spectral characteristics. The spectra of the influenza strains, of mumps virus, and of the normal host constituents were indistinguishable (curve A). Curve B represents the spectrum of NDV, which was closely related to curve A, except for the shape of the absorption pattern at about 1040 cm. $^{-1}$ (8.8 μ). All of the members of the psittacosis-lymphogranuloma venereum (LGV) group, which are antigenically related, gave the same spectra (curve C). The latter curve was identified by the absence of the shallow band at 1300 cm.$^{-1}$ (7.7 μ), the presence of a double hump at 1200 to 1130 cm.$^{-1}$ (8.3 to 8.8 μ) the reduced absorption of the broad band at 1100 to 1000 cm. $^{-1}$ (9.0 to 10.0 μ), and the exaggerated band at 960 cm. $^{-1}$ (10.4 μ). The shape of the curve at 1200 to 1130 cm. $^{-1}$ resembled that of NDV; however, the peaks

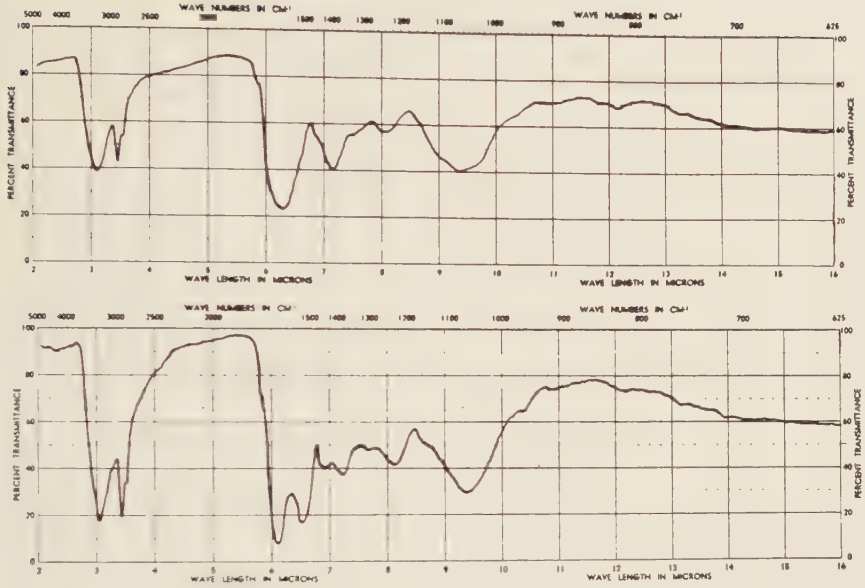


FIGURE 2. Spectra of normal host substances (top) and influenza A (bottom) prepared by one cycle of differential centrifugation.

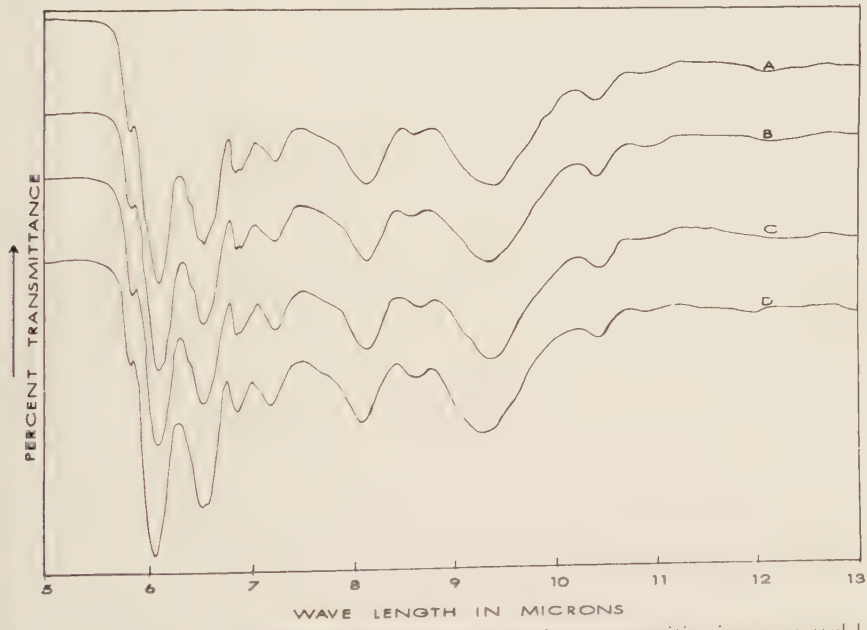


FIGURE 3. Spectra of separate preparations of meningopneumonitis virus processed by three cycles of differential centrifugation.

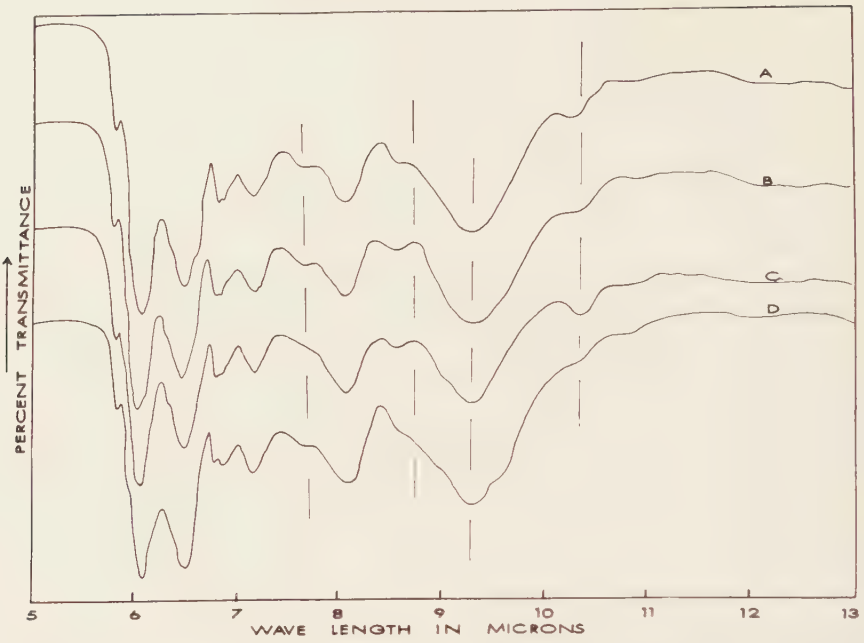


FIGURE 4. Spectra of virus preparations and of normal host components processed by three cycles of differential centrifugation: A, influenza A, A', B, mumps, and normal host components; B, Newcastle disease; C, psittacosis, lymphogranuloma venereum, meningopneumonitis, feline pneumonitis, mouse pneumonitis; D, vaccinia, fowl-pox. (Reproduced by permission from *Journal of Bacteriology*.[13])

of the humps were at the same height or slightly below the peak of the hump at 1350 cm.$^{-1}$ (7.4 μ). In the case of NDV, the vibrational region of 1200 to 1130 cm.$^{-1}$ was above the portion of the curve at 1350 cm.$^{-1}$ Vaccinia and fowl-pox preparations varied at 1280 cm.$^{-1}$ (7.8 μ), but the remainder of the spectrum was reproducible.

Subsequent to these studies, Kull and Grimm[11] investigated six serologically different *Bacillus megaterium* phages (M), purified tobacco mosaic virus (TMV), and *Escherichia coli* "T" phages. The same regions that were considered useful for the differentiation of the animal viruses were also used by these workers for the differentiation of the M and T phages. The subtle differences of the slope between 1350 and 1280 cm.$^{-1}$, which aided in the identification of curve C, served as a supplementary method for distinguishing closely related M phages. It was interesting to note that the following bands—1735, 1465, 1380, 1295, and 1175 cm.$^{-1}$—which were absent in the host bacterium were present in all spectra of the M phages and that the major bands of the bacterial cells showed more intense absorption in the M phage spectra (FIGURE 5).

The purification procedure used in our studies had reduced the influenza and mumps preparations to spectrally common components. These components may well represent host material, since identical spectra were obtained with normal AF. Whether they exist as contaminants or as integral parts of the

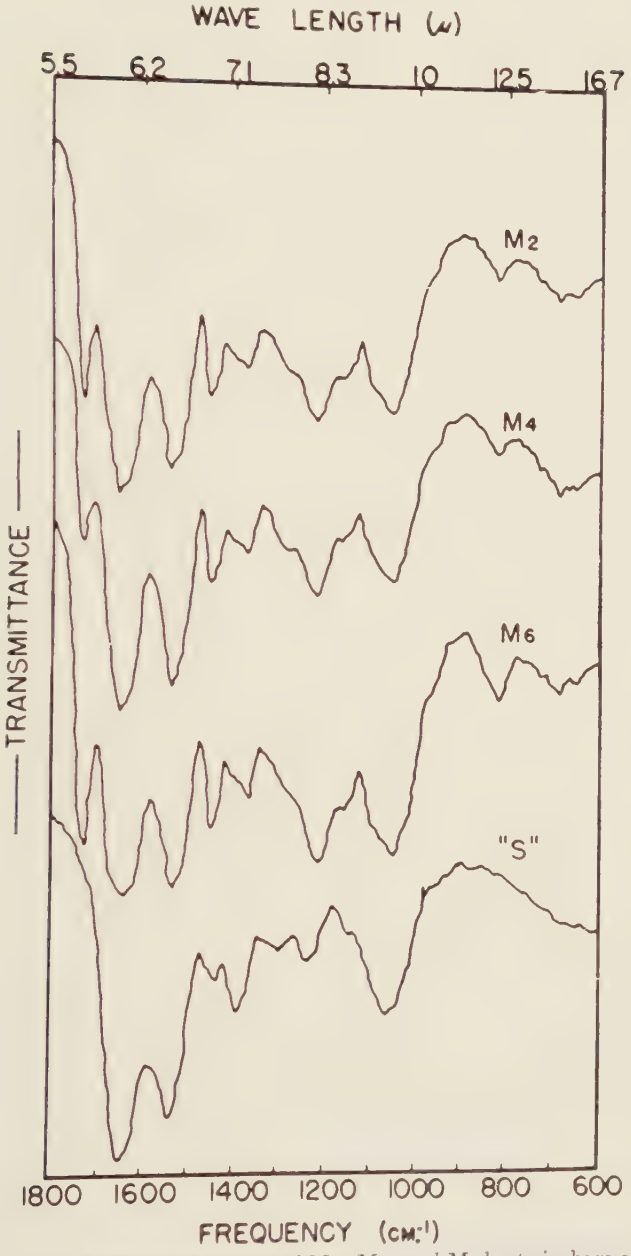


FIGURE 5. Infrared absorption spectra of M_2, M_4, and M_6 bacteriophage and host cell *B. megaterium* "S". (Reproduced by permission from *Virology*.[14])

virus particles is not known. Since the normal host, influenza, and mumps preparations showed the same spectra, variations in the host virus ratios would not be detected, as had been noted with less pure fractions. However, not all the viral preparations were as closely related to the host's structures (curves C and D of FIGURE 4). Because of the large particle size of the psittacosis-LGV viruses, purification was a relatively simple matter, and thus the higher concentration of virus particles perhaps masked the absorption due to host materials. Although host material was still microscopically detectable in the vaccinia and fowl-pox preparations, their unique curves probably represented the predominance of the virus particles. It should be mentioned that biochemically pure viruses were not studied and that host products resulting from infection, which are spectrally different from the normal host products, may contribute to the uniqueness of the spectra. Regardless of the constituents that were measured, related viruses have elicited the production of spectrally similar compounds.

Limiting the practical application of the infrared technique were the rigid conditions required to obtain reproducible spectra. Steps in the processing capable of altering the degree of purity or disrupting the integrity of the preparations caused some spectral changes. For example, repeated washing or repeated sequences of freezing and thawing of MP altered the 1220 to 1060 $cm.^{-1}$ (8.2 to 9.4 μ) vibrational region and attenuated the band at 960 $cm.^{-1}$ (10.4 μ) (FIGURE 6). The decreased absorption at 960 $cm.^{-1}$ probably was due to loss of nucleic acids, particularly as a result of leaching by repeated washings. Randall and Smith[15] reported that certain variations in the processing of the tubercle bacillus lipides did not have significant effects on the spectrum. Similarly, no changes in the spectrum were observed when psittacosis-LGV viruses were prepared from fluids obtained either from dead or living embryos, or when either 7- or 8-day-old chick embryos had been infected with these viruses and harvested after 12 days' incubation. In this connection, vaccinia virus preparations made from fluids harvested after 14 days' incubation gave spectra with a deeper band at 1100 to 1000 $cm.^{-1}$ (9.0 to 10.0 μ) than those obtained when the fluids were harvested from 12-day-old eggs.

Fortunately, the concentration of the viruses in the crude AF had no effect on the qualitative aspects of the spectra. Although LGV fluids had LD_{50} titers of 10^{-2} to 10^{-3}, in contrast to an LD_{50} titer of 10^{-5} for MP, the spectra of these two viruses were identical. When fluids contained low concentrations of viruses, it was necessary to process a large volume of fluid in order to secure a preparation meeting the criteria of the spectrophotometric technique. The lack of effect of concentration was supported further by the fact that when MP fluids of different known viral concentrations were processed, the spectra obtained were the same.

The method of purification employed served only as a screening technique and failed to delineate the members of each group. Indeed, the antigenic difference among related agents in the psittacosis-LGV group was sufficient evidence to propose physicochemical differences among these viruses. This emphasizes the fact that the infrared spectrum cannot be used as the sole criterion, and that evaluation of the chemical interpretation must of necessity

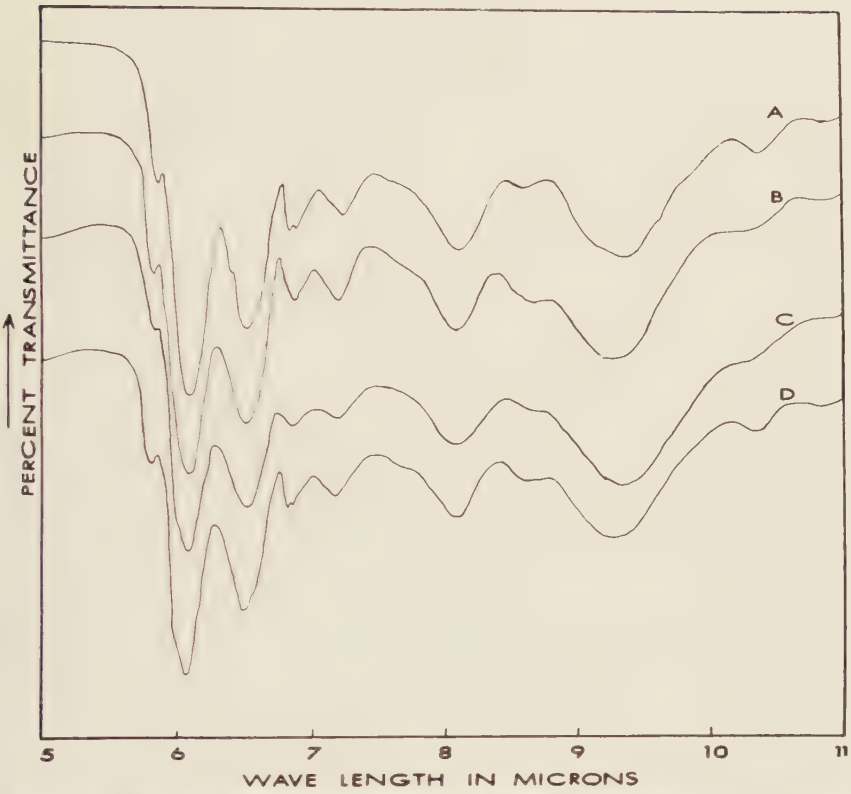


FIGURE 6. Effect of preparative variables on the spectra of meningopneumonitis preparations: A, processed by 3 cycles of differential centrifugation; B, processed by 7 cycles of centrifugation; C, subjected to repeated freezing and thawing; D, grown at 33 to 34° C. (Reproduced by permission from *Journal of Bacteriology*.[13])

include a combination of infrared spectroscopy and other physical and chemical means. In addition, a more complete classification of the viral types by infrared spectroscopy will be facilitated by methods that employ optimal purification and concentration techniques for the particular group of agents under consideration, or by extraction and purification procedures. The use of extraction overcomes the spectral neutralization due to the presence of diverse complex chemical structures, and hence will lead to a study of purer substances. We initiated this avenue of approach to a limited degree.

The absorption maxima for nucleic acids are between 1100 and 1000 cm.$^{-1}$ and at 960 cm.$^{-1}$ (10.4 μ)[16] and, as shown in FIGURE 4, the spectrum of MP virus had stronger absorption at 960 cm.$^{-1}$ than the spectrum of influenza virus. Extraction of nucleic acids from the influenza virus failed to produce appreciable changes in the broad band at 1100 to 1000 cm.$^{-1}$ or in the 960 cm.$^{-1}$ band, while extraction of MP virus caused decreased absorption at 1100 to 1000 cm.$^{-1}$ and disappearance of the 960 cm.$^{-1}$ band. The failure of the influenza-virus infrared spectrum to display changes following nucleic acid ex-

traction corroborates the recent findings of low contents of these substances in influenza viruses: the concentrations of ribonucleic acid (RNA) are about 0.7 to 0.8 per cent and of deoxyribonucleic acid about 0.1 per cent.[17-19] In contrast, appreciably higher nucleic acid concentrations for MP virus can be approximated,[20, 21] and reflection of this moiety was evident in the spectra of the MP preparations. Under such conditions tentative interpretation of the spectra can be made.

In the light of the specificity of the spectra of the lipides of tubercle bacilli,[15] studies were undertaken to determine whether lipide fractions could be used for identification of the viruses. Three members of the psittacosis-LGV group—MP, psittacosis, and feline pneumonitis viruses were extracted with either acetone, methanol, or ether, but neither the residues nor the extracts were sufficiently reproducible for identification purposes. Methanol-chloroform extracts did yield reproducible spectra, but the spectra of the extracts of the three viruses were identical. The major changes resulting from extraction were attenuation of the C –H groups at 2940 to 2860 cm.$^{-1}$ (3.4 to 3.5 μ) and disappearance of the band at 1720 cm.$^{-1}$ (5.8 μ) (FIGURE 7); the latter band is probably due to the presence of undissociated carboxyl groups or carbonyl groups.[22] It would appear that one of the identifying characteristics of the psittacosis-LGV viruses, namely the absorption at 1250 to 1100 cm.$^{-1}$ (8.0 to 9.0 μ), may be due in part to their lipide content. Following methanol-chloroform extraction the spectra of the MP residue resembled those of group A (FIGURE 4). This same region in the spectrum of the lipide fraction showed intense absorption. The other identifying characteristics of MP (1300, 1100 to 1000, and 960 cm.$^{-1}$) were not altered by extraction with lipide solvents.

A striking difference among the animal viruses, bacterial viruses, and TMV

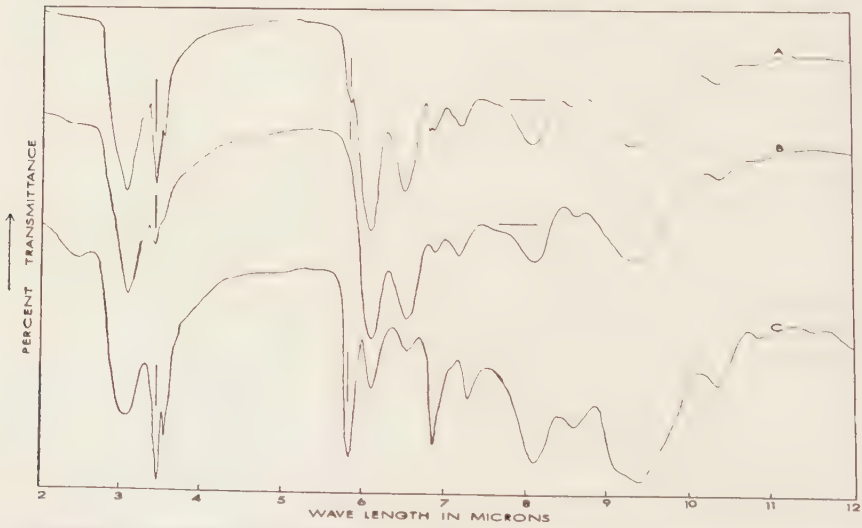


FIGURE 7. Extraction of meningopneumonitis preparations with methanol chloroform: A, before extraction; B, residue after extraction; C, extract. (Reproduced by permission from *Journal of Bacteriology*.[13])

lies in the 1720 cm. $^{-1}$ (5.8 μ) band of their spectra. The animal viruses all showed similar absorptions at this wave number, whereas Kull and Grimm[14] found increased absorption at 1720 cm. $^{-1}$ by the M phages and the absence of this band from the spectra of the host cell, of TMV, and of T_3 and T_4 *E. coli* phages. These investigators suggested that substances responsible for the absorption at 1720 cm. $^{-1}$ probably were neutralized by stronger nitrogen influences exhibited by nucleic acid. This idea is supported by our studies with fowl erythrocytes,[28] in which, in spite of the lipide esters, little or no absorption was noted in the 1720 cm. $^{-1}$ region and the nucleic acid constituents constituted a dominant feature of the spectrum.

The MP virus residue remaining after solvent extraction was extracted with cold 10 per cent trichloroacetic acid (TCA) and ethanol to remove the polysaccharides.[24] The residue remaining from this treatment showed less absorption at 1410, 1230, and 1100 to 1000 cm. $^{-1}$ (7.1, 8.1, and 9.0 to 10.0μ) (FIGURE 8), thus implying that these bands were due partly to the polysaccharides. Extraction of this residue with hot 5 per cent TCA[24] further attenuated the 1230 cm. $^{-1}$, 1100 to 1000 cm. $^{-1}$, and 960 cm. $^{-1}$ (10.4 μ) bands, suggesting that nucleic acids were partly responsible for them. These spectra were similar to those obtained previously by Levine *et al.*[25] with *E. coli* extracted in a similar manner.

It was felt that a general application of infrared spectroscopy in virology would be that of testing the utility of the spectrum as a guide for control of

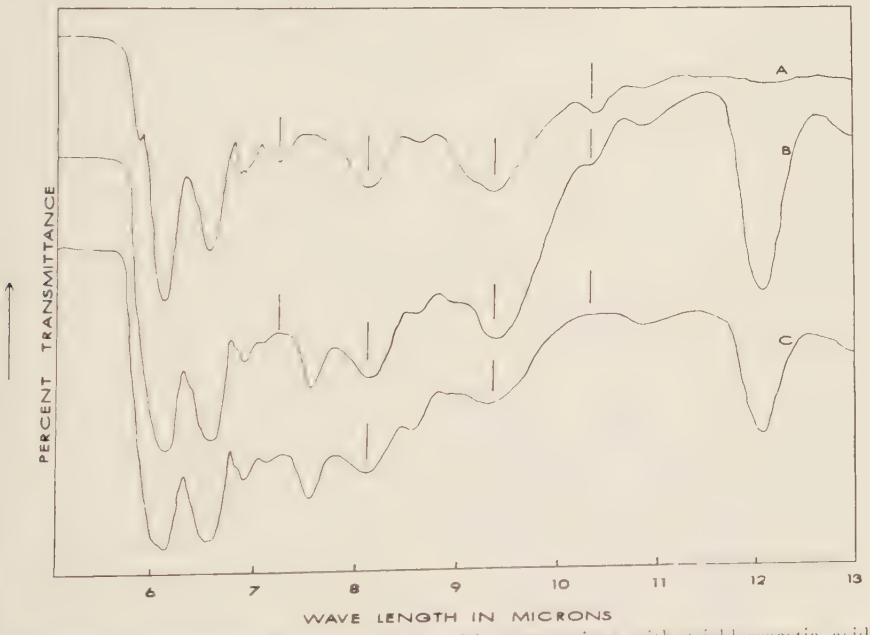


FIGURE 8. Extraction of meningopneumonitis preparations with trichloroacetic acid (TCA): A, before extraction; B, residue after extraction with cold 10 per cent TCA; C, residue after extraction with hot 5 per cent TCA. (Reproduced by permission from *Journal of Bacteriology*.[13])

TABLE 1
BASIC CHEMICAL DATA ON THE MENINGOPNEUMONITIS GROUP COMPLEMENT-FIXING ANTIGENS

	Antigens*		
	Acid-precipitated	Phenol-insoluble	Phenol-soluble
Total N (%)	4.8	2.8	10.1
Reducing sugars after hydrolysis (%)	20.0	42.5	<1
Methanol-chloroform-soluble fraction (lipide) (%)	33	55	~0
Phosphorus (%)	1.6	0.8	—
Deoxyribonucleic acid	Negative	—	—
Molisch	Strong	Strong	Negative
Biuret	Weak	Negative	Weak

* Prepared from virus suspensions purified by 5 cycles of differential centrifugation.
Symbol: —, not tested.

fractionation and for the identification of biologically active materials. In this regard, infrared proved to be a valuable adjunct for the chemical elucidation of two psittacosis complement-fixing (CF) antigens.[26] A CF antigen, which was extracted from MP with a surface-active agent, was partially purified by repeated acid precipitations. The basic chemical data, summarized in TABLE 1, revealed a protein-polysaccharide-lipide complex. Treatment of this complex with 5 per cent neutral phenol produced a periodate-sensitive phenol-insoluble antigen, and a periodate-resistant phenol-soluble antigen. The phenol-insoluble fraction had a lower nitrogen content (TABLE 1), increased reducing-sugar and lipide values, and increased infrared absorption at 1720 cm.$^{-1}$ (5.8 μ) and 1230 cm.$^{-1}$ (8.1 μ) (FIGURE 9). These bands are indicative of C=O and

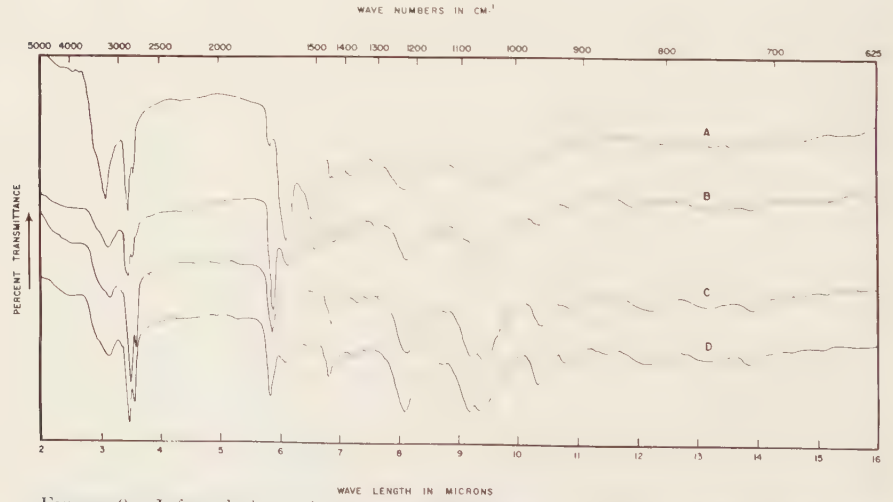


FIGURE 9. Infrared absorption spectra of MP complement-fixing antigenic fractions: A, crude antigen reprecipitated with acid five times; B, phenol precipitate; C, methanol-chloroform extract (lipide) of phenol precipitate; D, lecithin.

C—O bonds, respectively, which may be contributed by both the lipide and polysaccharide moieties.[22] Absorption peaks at about 1650 cm.$^{-1}$ (6.05 μ) and 1540 cm.$^{-1}$ (6.5 μ) are characteristic monosubstituted amide bands[22] and, in view of the inability of the infrared technique to detect protein in a preparation giving negative protein tests, these bands were probably of polysaccharide origin. On the basis of minimal chemical analysis coupled with the infrared spectrum, the data strongly suggested that the phenol precipitate was a serologically active lipocarbohydrate.

With the phenol supernatant fraction poorly differentiated spectra were obtained. However, strong absorption was obvious at 1650 and 1550 cm.$^{-1}$, and no band was seen at 1720 to 1790 cm.$^{-1}$ (5.6 to 5.8 μ). The antigen is believed to be chiefly protein, since neither lipide nor carbohydrate was detected by chemical analysis.

Methanol-chloroform extracts of the phenol precipitate yielded a spectrum similar to that of lecithin. The spectrum for cholesterol was determined and showed different maxima from those shown by the antigen lipide fraction. According to published spectra[27] the cephalins do not have a distinct band at 970 cm.$^{-1}$ (10.3 μ), and sphingomyelin has amide bands at 1640 and 1540 cm.$^{-1}$ (6.1 and 6.5 μ) rather than the carbonyl band at 1720 cm.$^{-1}$

In summary, there are certain problems related to the study and identification of viruses by infrared spectroscopy. Because of varying degrees of host contamination, highly impure preparations of animal viruses do not give reproducible spectra. Furthermore, only under specific conditions were reproducible spectra obtained, and then the twelve agents studied formed only four spectral groups. Closely related viruses were not distinguished, thus suggesting that a greater degree of purity was required. This purification must be effected either by the monumental task of eliminating contaminating host substances, or by unmasking, by extraction procedures, fractions that can be used for identifying purposes.

It is admitted that, in most instances, we are only beginning to obtain pure animal virus preparations for such studies, although the outlook is bright in view of the recent progress on the purification of the poliomyelitis[28] and other viruses. The work reported here reveals that, if one is aware of the limitations of the method, corroborating chemical data on whole virus preparations or fractions thereof can be obtained with the infrared spectrum.

References

1. Barer, R., A. R. H. Cole & H. W. Thompson. 1949. Infra red spectroscopy with the reflecting microscope in physics, chemistry and biology. Nature. **163:** 198–201.
2. Blout, E. R. & R. C. Mellors. 1949. Infrared spectra of tissues. Science. **110:** 137–138.
3. Schwarz, H. P., C. Glick, W. Cameron, E. Beyer, B. Jaffe & L. Trombetta. 1951. Infrared spectroscopy of tissues. Effect of insulin shock. Proc. Soc. Exptl. Biol. Med. **76:** 267–272.
4. Stevenson, H. J. R. & O. E. A. Bolduan. 1952. Infrared spectrophotometry as a means for identification of bacteria. Science. **116:** 111–113.
5. Simon, S. & L. R. Hedrick. 1955. Infrared spectrophotometry of Hansenula and Saccharomyces whole yeast cells and yeast cellulose. J. Bacteriol. **69:** 4–8.
6. Randall, H. M., D. W. Smith, A. Colm & W. J. Nungester. 1951. Correlation of biologic properties of strains of *Mycobacterium* with their infrared spectrums. I.

Reproducibility of extracts of *M. tuberculosis* as determined by infrared spectroscopy. Am. Rev. Tuberc. **63:** 372–380.
7. Fraser, R. D. B. & J. Chayen. 1952. The detection of nucleic acid in tissues by infrared microspectrometry. Exptl. Cell Research **3:** 492–493.
8. Levine, S., H. J. R. Stevenson, E. C. Tabor, R. H. Bordner & L. A. Chambers. 1953. Glycogen of enteric bacteria. J. Bacteriol. **66:** 664–670.
9. Schwarz, H. P., H. E. Riggs, C. Glick, J. McGrath, R. Childs, E. Bew, Jr. & F. Stone. 1954. Infrared spectroscopy of liver glycogen in normal and irradiated adult and fetal rats. Proc. Soc. Exptl. Biol. Med. **85:** 96–101.
10. Astbury, W. T. & N. N. Saha. 1953. Structure of algal flagella. Nature. **171:** 280–283.
11. Pollard, M., F. B. Engley, Jr., R. F. Redmond, H. I. Chinn & R. B. Mitchell. 1952. Infrared absorption spectra of viruses. Proc. Soc. Exptl. Biol. Med. **81:** 10–11.
12. Benedict, A. A., M. Pollard & F. B. Engley, Jr. 1954. Infrared absorption studies of virus preparations. Texas Repts. Biol. Med. **12:** 21–29.
13. Benedict, A. A. 1955. Group classification of virus preparations by infrared spectroscopy. J. Bacteriol. **69:** 264–269.
14. Kull, F. C. & M. R. Grimm. 1956. Infrared absorption spectra of *Bacillus megaterium* phages and host cell. Virology. **2:** 131–138.
15. Randall, H. M. & D. W. Smith. 1953. Infrared spectroscopy in bacteriological research. J. Opt. Soc. Am. **43:** 1086–1092.
16. Blout, E. R. & M. Fields. 1948. On the infrared spectra of nucleic acids and certain of their components. Science. **107:** 252.
17. Ada, G. L. & B. T. Perry. 1954. The nucleic acid content of influenza virus. Australian J. Exptl. Biol. Med. Sci. **32:** 453–468.
18. Miller, H. K. 1956. The nucleic acid content of influenza virus. Virology. **2:** 312–320.
19. Frommhagen, L. H. & C. A. Knight. 1956. The polysaccharide and ribonucleic acid content of purified influenza virus. Virology. **2:** 430–431.
20. Crocker, T. T. 1952. Estimates of particle number, lethal effect, and chemical properties of virus of meningopneumonitis. Federation Proc. **11:** 464–465.
21. Zahler, S. A. & J. W. Moulder. 1953. The incorporation of radioactive phosphate into feline pneumonitis virus in the chick embryo yolk sac. J. Infectious Diseases. **93:** 150–165.
22. Randall, H. M., R. G. Fowler, N. Fuson & J. R. Dangl. 1949. Infrared determination of organic structures. Van Nostrand. New York, N. Y.
23. Benedict, A. A. 1954. Infrared absorption studies of fowl and mammalian erythrocytes. Exptl. Cell Research. **7:** 565–567.
24. Schneider, W. C. 1945. Phosphorous compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. **161:** 293–303.
25. Levine, S., H. J. R. Stevenson, L. A. Chambers & B. A. Kenner. 1953. Infrared spectrophotometry of enteric bacteria. J. Bacteriol. **65:** 10–15.
26. Benedict, A. A. & E. O'Brien. 1956. Antigenic studies on the psittacosis-lymphogranuloma venereum group of viruses. II. Characterization of complement-fixing antigens extracted with sodium lauryl sulfate. J. Immunol. **76:** 293–300.
27. Freeman, N. K., F. T. Lindgren, Y. C. Ng & A. V. Nichols. 1953. Infrared spectra of some lipoproteins and related lipids. J. Biol. Chem. **203:** 293–304.
28. Schwerdt, C. E. & F. L. Schaffer. 1956. Purification of poliomyelitis viruses propagated in tissue culture. Virology. **2:** 665–678.

INFRARED SPECTRAL STUDIES OF TISSUES*

By Leopold May and R. G. Grenell
The Psychiatric Institute, University of Maryland, Baltimore, Md.

Infrared absorption spectrophotometry has provided a method for the identification and quantitative analysis of chemical entities in relatively simple mixtures. One of the basic assumptions of the work to be discussed in the present review is that it also has been presumed to serve the same function in the analysis of the chemical constituents of highly complex mixtures such as are found in living cells. Investigators with widely differing degrees of sophistication as to the full implications of the problem have approached it in the hope that measurements of variations or alterations in molecular structure would be observable, identifiable, and quantifiable in different areas of a tissue as well as from one tissue to another. If the technique of recording molecular vibrations permits one to study bond changes occurring in the basic mechanisms of the functional activity of cells with the necessary degree of clarity and accuracy, the value of such a technique is obvious. One of the aims of this review is to assess critically what has been done in an effort to reveal the most fruitful course to be pursued as well as to evaluate the present status of both infrared technique and concept in biology.

For these reasons this summary will include discussions of: (1) the various methods of sampling living and fixed cells; and (2) the data and ancillary problems resulting from absorption and reflectance studies of tissues, cells, and subcellular structures.

Previous reviews of infrared spectrophotometry for biologists have included some of the results of tissue investigation.[1-4]

Techniques

The principal difficulty in obtaining spectra of living tissues is the large ratio of water to solids in the cellular protoplasmic suspension and the intense absorption of water in the 3.0 μ and 6.0 μ regions. The use of an aqueous solution in the compensating beam of the spectrometer has permitted the recording of spectra of muscle fibers.[5] Blood has been examined, and the absorption due to water has been partially compensated for by the use of screens.[6] The infrared cells were prepared from BaF_2 and AgCl. Various cell materials have been proposed for aqueous solutions, although many have a limited spectral range of usefulness.[7] The near infrared region does not present this difficulty since water does not absorb to any great extent below 1.4 μ. In some cases D_2O has been substituted for ordinary water. Under these circumstances possible deuterization of certain groups must be considered in the interpretation of the spectrum.[8-10]

Tissue sections have been dehydrated and fixed in various ways. The fixa-

* The work on which this paper is based was supported by Contracts N7-onr 39706 and N7-onr-59503 between the Office of Naval Research, Washington, D. C., and the University of Maryland, and Grant M 795 (C2) from the National Institute of Mental Health Public Health Service, Bethesda, Md.

tion is usually done with chemicals such as formalin or Carnoy's solution or by quick freezing. There is ample histochemical evidence to demonstrate that any form of chemical fixation can (and does) alter molecular structure, as well as the distribution and amount of proteins and other substances in the cell. Only extremely careful freeze-drying of very small pieces of tissue preserves the major portion of the living chemical pattern. After fixation with chemicals the tissue is embedded in paraffin. It is desirable to remove the paraffin before the spectrum is measured; otherwise, interferences will occur in the regions of strong absorption of paraffin. The removal of the paraffin necessitates the use of xylene and absolute alcohol, which are lipide solvents. The use of quick freezing and subsequent removal of water by placing the frozen section in a vacuum eliminates the use of paraffin.[11, 12] Blout and Mellors[13] note that an inflection point at 3070 cm.$^{-1}$ appears in the spectra of carcinoma tissues prepared by formalin fixation, but not in the spectra of sections that have been frozen and then dehydrated. However, this band has been consistently observed in frozen sections of nervous tissue[11] and of muscle.[14] Several absorption bands appeared in the spectrum of a frozen section of human mammary carcinoma, but not in the spectrum of the formalin section;[15] in addition, a band at 1047 cm.$^{-1}$ was shifted toward longer wave lengths. These changes may be related to the removal of certain lipide components by xylene in the course of removing paraffin from the formalin-fixed sections.

Scattering of the infrared rays is a major problem in the determination of the spectra of tissues, as with all solids. It can be reduced by the use of a hydrocarbon oil, such as Nujol, spread over the section.[13] This introduces the absorption bands of the oil at 3, 7, and 13.8 μ. The use of other materials such as perfluorolube oil permits the recording of spectra without interference in the 1 to 7.3 μ region.[13] Another procedure that has been used consists of comparison of a plate containing the sample and a blank in the 10 μ region where no absorption band appears. The blank is ground until the transmittance of the sample plate is nearly 100 per cent.[15] Thomas and Greenstreet[16] obtained reproducible spectra of dried films of bacteria in a double-beam spectrophotometer by adjusting the reference beam with an iris diaphragm until the sample showed about 95 per cent transmittance at both 5.5 and 11.5 μ, where no absorption bands appear.

Homogenization of tissues eliminates the problem of chemical fixation involved in the preparation of tissue sections. However, it does alter the morphology and makes it impossible to obtain infrared spectra of localized areas within the cell. The homogenate is prepared as a film on AgCl or NaCl plates. For nervous tissue, both the homogenate films and tissue sections have qualitatively the same spectra.[11, 17] The homogenate can be lyophilized and then prepared as a film for spectral analysis. Films of lyophilized nervous tissue have the same spectra as tissues.[11]

Another technique for the examination of solid samples is the use of disks prepared by pressing the sample with potassium halide salts such as KBr and KCl.[18, 19] Cook *et al.*[20] presented a spectrum of rabbit serum prepared as a KBr disk, but did not include any comparison with rabbit-serum spectra obtained in any other manner. Since it was observed that the spectrum of

thiourea in a KBr disk is different from that of the Nujol film,[21] it is advisable to check the spectra of tissues prepared as disks and those obtained from tissue prepared by one of the other methods. For nerve tissue the positions of the absorption bands are the same for sections, homogenates, lyophilized films, and KBr disks.[22] These disks have the advantages of permitting the recording of spectra without the interference of the absorption bands of mulling agents, of reducing the scattering at short wave lengths, of requiring smaller quantities of the sample, and of permitting the storage of specimens without tying up relatively expensive cell materials such as AgCl or BaF_2. In addition, quantitative chemical ultramicroanalyses can be performed on the sample within the disk and can be related to the absorption found both in the infrared and ultraviolet regions.

Extraction procedures have been employed to aid in the identification of various chemical constituents in the cell. These involve the removal of certain constituents through the use of different solvents. It is desirable to record the spectrum of the tissue prior to extraction so that any change in the tissue spectrum due to an altered physiological state can be related to changes in the spectra of the extracted materials. In addition, the spectra of both the extractant and the residues should be correlated with information obtained by other methods—chemical, optical, chromatographic, and the like. The uses of aqueous salt solutions would permit fractionation of the protein constituents in a "native" state. This would allow examination of changes in the structure of macromolecular components of the cell with changes in the physiological state of the cell. The interfering salts used in the fractionation could be removed by standard biochemical procedures, such as dialysis, and the spectra of the protein fractions prepared as films, disks, or even as aqueous solutions could be recorded. Another type of extraction involves the use of enzymes, as in the isolation of lignin from white Scots pine, using molds.[23]

The various techniques for recording the spectra of cells may yield different spectra. In water solution, the effect of *p*H may alter the position of certain bands that arise from ionizable groups. This variable should be controlled when a study is made of aqueous extracts. The use of D_2O involves both this factor and the effect of deuterization on the spectra. The methods of preparing tissue for spectral analysis involve processes such as destruction of the cellular integrity (as in homogenization) and alteration of the structure of different constituents (as in lyophilization). This may not be evidenced by variation in the position of the absorption bands, but in the absorptivities of the constituents contributing to these bands.

Tissues

Plants. Absorption studies have been reported for various woods[23-26] and for homogenized wood.[27] Kratzl and his co-workers[25, 26] compared the spectra of various substances such as 6-tritylcellulose, furfural, and lignin with the spectra of spruce wood and various extracts. They concluded that the spectrum of whole wood combines the features of carbohydrate and of lignin containing considerable amounts of aromatic nuclei.

Nord and his co-workers[25, 29] examined the spectra of native lignins extracted

with alcohol from pine, birch, oak, maple, and cork. Differences were observed in the intensities of most bands, and pronounced bands were present only at 870 and 890 cm. $^{-1}$ in the spectrum of maple lignins. They[29] quote the work of Jones[30] as evidence for the presence of bands in the spectrum of spruce lignin in this region. Jones's spectrum, however, was determined with a film prepared from a dioxane solution that also absorbs in this region (Kudzin and Nord[29]). Jones[31] published spectra of native spruce lignin prepared from dioxane-ethyl alcohol solution and as a Nujol mull. In both spectra, a band appears at about 890 cm.$^{-1}$ The spectrum of native aspen lignin is quite different from that of native spruce lignin.[32] The band at 870 cm.$^{-1}$ appears in the spectra of various fractions of cork lignin, but not in the native lignin spectrum.[28] Jones[32] fractionated native spruce lignin and found variations of the C=O bonding in the various fractions. The enzymatic extraction[25] of lignin from white Scots pine using mold produced a lignin that had the same spectrum as lignin isolated from undecayed wood.

Gates and Tantraporn[33] measured the reflectance from leaves of numerous deciduous trees and shrubs in the region to 25 μ. The degree of roughness of the surface influences the reflectance of infrared beyond 1 μ. The upper surface of *Quercus robur* is smooth, but the lower surface is granulated. This latter surface reduced the reflectance by a factor of 2.0. The light is mostly reflected at the outer epidermal surface. Old leaves reflect more than young, and the shade leaf more than the sun leaf. The whole leaf transmitted no light of a wave length greater than 1 μ, but the transmittance of the epidermis is about 40 per cent. Absorption bands appear at 1.4, 1.93, and beyond 2.5 μ in the spectra of the components of buds of *Tradescantia paludosa*. These bands are assigned to the absorption of liquid water in this region.[34]

Herbst[35] studied the spectra of various extractants of healthy potatoes and of potatoes infected with mosaic virus and "Hollander Erstling" disease. Differences were found between the spectra of diseased and healthy plants. In the chloroform-methanol extract, for example, additional bands were observed for the diseased plants at 917, 870, and 775 cm.$^{-1}$ The spectra of potatoes attacked by viruses showed an increase in bands attributed to P-groups and to nucleic acids. Herbst concluded that further experimentation is necessary before an evaluation of infrared spectroscopy in the determination of the seed value of potato tubers can be made.

Stair and Coblentz[36] measured the spectra of membranes from pokeweed pith, onion skin, seed wing, and the seed septum from the seed pod of moonwort. They compared these spectra with the spectrum of cellophane and concluded that there was a close resemblance. With many samples, the specimens were too thick to permit further characterization. Fraser and Chayen[37] correlated certain absorption bands in the spectrum of bean root tip with the bands found in the spectrum of ribonucleic acid.

Microorganisms. The infrared spectra of whole bacterial cells have been examined in an effort to provide a method for the identification of individual species. Stevenson and Bolduan[38] prepared dried films from colonies grown on agar plates. They observed that, although the thickness of various preparations of *Serratia marcescens* varied and the spectral curves were not identical,

they were qualitatively the same. Thomas and Greenstreet[16] confirmed this observation. It was noted that variation in the culture medium and time of incubation produced changes in the spectra.[16, 39] However, Simon and Hedrick[40] state that the spectra of *Hansenula anomala* whole yeast cells and of isolated cellulose were "similar" even though the culture medium was changed. The effect of variation of the time of incubation was not recorded. Stevenson and Bolduan[38] found it necessary to control culture medium, age of culture, and temperature of incubation, since these variables changed the spectra of different species in varying degrees. Kabler *et al.*[41] studied the spectra of 142 strains of coliform and related bacteria. Their results indicated that differentiation of every species and serologic group could be made from the spectra. Although differentiation could be made among various species, strains of a single species sometimes gave the same spectra.[16, 38] Levine *et al.*[42] in their study of *Klebsiella* stated that the spectra of whole cells had little value in typing these organisms. Kull and Grimm[43, 44] studied the spectra of various bacterial species and their strains that were made resistant to various antibiotics and antituberculous compounds. With some resistant strains, new bands appeared at 835, 1176, and 1735 cm.$^{-1}$, but in others no change was observed. They concluded[45] that "infrared as a qualitative differentiating tool is limited if crude extracts, whole or intact cells are used."

Attempts to adopt a quantitative method using absorbance ratios between different bands and the band at 1550 or 1667 cm.$^{-1}$ failed.[44] These bands were arbitrarily assigned the value of 100 per cent absorption. The band at 1550 cm.$^{-1}$ has been used as a measure of the film thickness by Stevenson and Bolduan.[38] This method of calculation should eliminate the effect of film thickness, but it must be recalled that the concentration of the substances contributing to the absorption at these bands (1550 or 1667 cm.$^{-1}$) could be different with the different resistant strains. Certain resistant strains have a new band at 1735 cm.$^{-1}$ that might be presumed to be characteristic of lipide components of tissue since lipide components with amide groups, such as cephalin, have absorption bands in this region.[46] This method does not compensate for light scattering by the cells. The method of Levine *et al.*,[47] in which the band at 1550 cm.$^{-1}$ is used as an internal standard, also did not provide means for quantification with whole bacterial cells.[44] The method is also subject to the same criticism noted for the other quantitative method.

Blout and Lenormant[9, 48] published the spectra of living bacteria in D_2O. They stated that the spectrum resembled that of a nucleoprotein with a predominance of ribonucleic acid.[48] It was suggested that the differences in the spectra of different species of bacteria might be caused by varying amounts of the different nucleic acids. Levine *et al.*[39] studied the spectra of various extracts and fractions of enteric bacteria and concluded that the band near 1250 cm.$^{-1}$ was due mainly to nucleic acid and that the broad band at 1130 cm.$^{-1}$ had contributions from the absorption of nucleic acids and polysaccharides. Levine *et al.*[47] identified the absorption bands characteristic of glycogen in the whole bacterial cell and used the ratio of the absorbances at 1026 and 1550 cm.$^{-1}$ as a semiquantitative measure of the glycogen concentration in enteric bacteria.[49] Support for the validity of this technique was afforded by direct

biochemical determinations. They observed that the glycogen content is influenced by the culture conditions.

Another approach to the problem of identifying different species and strains of bacteria involves the spectral characterization of various extracts. Smith and his co-workers have been studying the lipide fractions of *Mycobacterium tuberculosis* and their work has been summarized.[50] Levine and his co-workers[51, 52] extracted polysaccharides and showed that the infrared spectrum could be used to differentiate 57 types and subtypes of *Pneumococcus* except for one pair. The spectra of crude extracts of polysaccharides from various types of *Klebsiella* were shown to be useful in typing these bacteria.[42] Schneider and McLaughlin[53, 54] concluded from a study of various fractions of different strains of *Leptospira* that infrared spectroscopy appeared to have potential value as a supplement to the classical serologic methods. Levi *et al.*[55] determined the spectra of cold ethanolic extractions from seven strains of *Micrococcus pyogenes*. Weighed samples of the extract were prepared as KBr disks. It was found that the area of the spectral curve from 1000 to 1100 cm.$^{-1}$ was always higher for preparations that were biologically inactive.

Shirk and Greathouse[56] identified purified bacterial films harvested from *Acetobacter xylinum* to be cellulosic in nature from their infrared spectra. Slight variations in the spectra were noted with different media.

Astbury and Saha[57] found that there was a general resemblance between the spectra of *Chlorogonium flagella* and stretched horsehair, whose spectrum is characteristic of β-keratin. Acidification of the flagella revealed in the spectra new bands that were characteristic of carboxyl groups and the relative abundance of which was suggested as the basis for the difference between these two specimens. The absorption bands in the flagella spectrum in the region 950 to 1200 cm.$^{-1}$ were similar to those found with carbohydrates.

Insects. Coblentz[36, 58, 59] investigated the spectra of the outer coverings of fireflies and other insects. There was no transmission of light in the 1 to 2 μ region or above 5 μ. The spectra were said to be characteristic of complex carbohydrate even though other regions of the infrared spectra were not examined. Rücker[60] found broad absorption bands at 3 and 6 μ in the spectra of the elytra of several insects. Micks and Benedict[61] homogenized mosquitoes in distilled water, and the infrared spectra were measured with dried films of the supernatant solutions. In the region 6 to 13 μ, the differences observed between species were restricted to differing relative intensities of the bands at about 1440 and 1390 cm.$^{-1}$ and the relative depths and shapes of the broad band from 1000 to 1160 cm.$^{-1}$ Beyond this, no organized attempt has been made to investigate this area.

Skin. Rücker[60] studied the infrared absorption of the skins of various reptiles, amphibians, and snails in the region of 1 to 10 μ, and found absorption at 3 and 6 μ. He concluded that the shell of most snails provides protection against infrared radiation, but that *Hilicella obvia* may be an exception. Krüger[63, 64] measured the absorption spectrum of the skin of reptiles and found absorption at 3 μ and at 6 μ, the maximum wave length he could examine.

The transmission of light in the near infrared of living flesh was measured in the human cheek by Cartwright.[65] Maximum transmittance was found at 1.1

μ, and there was complete absorption above 1.4 μ, where water absorbs. Bacon fat had similar spectral characteristics. Forsythe and Christison[66] corrected the transmittance curve of Cartwright by 34 per cent, which is the amount of radiation reflected; however, this did not alter the shape of the curve. Bachem and Reed[67] measured the near-infrared absorption spectra of epidermis from various parts of the human body. They concluded that the far infrared has very little penetrating power because most of it is absorbed in the epidermis. It must be noted, however, that many of the preparations included several layers of living tissue deep to the surface.

Lenormant[68] measured the spectrum of frog epidermis in the region between 5.5 and 7.5 μ. There is good agreement between this spectrum and the spectrum of human epidermis measured by Hardy and Muschenheim,[69, 70] who compared the spectra of wet and dry epidermis. The spectrum of the dry specimen has sharp bands, and that of the wet sample has regions with generalized absorption and the absorption bands of water. The near infrared spectra (1 to 2.4 μ) of the ears of both dead and living rabbits, as well as of human skin, have the same general characteristics. These investigators concluded that the "spectrum of normally wet skin is essentially that of liquid water." Although this generalization overlooks certain spectral characteristics, there can be no strong opposition to the finding that the spectrum of water looks like itself.

Clark, Vinegar, and Hardy[71] described a procedure for measuring the transmittance, absorbance, and reflectance of skin simultaneously, using a goniometric spectrometer in the region of 0.5 to 2.5 μ. For human white breast skin, the reflectance increases at the wave lengths where absorption appears. Hardy *et al.*[72, 73] used this equipment on excised skin and concluded that the reflectance and transmittance spectra are identical for all types of skin for wave lengths greater than 1 μ. Beer's Law is obeyed in this region, but not between 0.5 and 0.95 μ, where the presence of pigment influences the values obtained.

Kuppenheim and his co-workers[74-77] measured the reflectance of skin in the region of 0.7 to 2.6 μ. Similar spectral reflectance curves were shown by the skin of all species examined: humans of different colors (Caucasians, Japanese, Negroes), Chester White pigs, rabbits, rats, and hairless mice. These investigators quote the results of Krolak and Davis,[78] who obtained similar reflectance curves for the skin of the pig. Alpen *et al.*[79] measured the critical energy for "minimal white" burn production and the reflectance of human and rat skin in the region 0.3 to 2.5 μ using filters. The reflectivity did not account for the variation of the critical energy with wave length. It was suggested that the transmission properties of skin may be of some importance since high critical energies were observed in the region of maximum transmittance (*ca.* 1.0 μ).

In most reflectance work, a standard material such as magnesium carbonate or magnesium oxide is used, and all values are reported relative to the standard. Since the reflectance of skin depends upon texture as well as on thickness, the angle of incidence, and the wave length of the radiation, the measurements involving a standard may be in error by an unknown amount. The goniospectrometer[71] permits measurement of absolute values.

Osteoid Tissues. Bone and teeth consist of inorganic crystalline material embedded in an organic matrix. Caglioti, Ascenzi, and Scrocco[82, 83] compared the infrared absorption spectra of whole bone, ossein (organic matter), and the inorganic phase of ox femoral diaphysis. The band at 1213 cm.$^{-1}$ in ossein spectra decreases in intensity in the spectra of whole bone, but the absorption at 2155 cm.$^{-1}$ disappears. It is proposed that these bands are related to SO_2 and that the band at the higher frequency is the first overtone of the fundamental. This explains the disappearance of the latter band with the weakening of the lower frequency band. The band near 1000 cm.$^{-1}$ is assigned to orthophosphate.[82-84] The spectra of the inorganic fractions of dentine, enamel, and bone prepared as KBr disks[84, 85] are the same as the spectrum of this fraction from bone examined as a powder. However, the spectra of the KBr disks give sharper bands. Caglioti, Ascenzi, and Scrocco[82, 83] stated that the band near 850 cm.$^{-1}$ is related to PO_4 ion, but Posner and Duyckaerts[84] assigned this band to carbonate by comparison with calcium and magnesium carbonates. Underwood, Toribara, and Neuman[85] concluded from a comparison of tissue, carbonate, and bicarbonate spectra that carbon dioxide exists entirely as the carbonate in these tissues. Posner and Duyckaerts[84] suggested that there exists a chemical bond between calcium and carbonate and magnesium and carbonate in tissue and that this bond is identical to the bonds in calcite and magnesite. McConnell[86] objected to this on the basis that the infrared data presented "cannot possibly yield information concerning the configuration of other atoms in the vicinity of the CO_3 groups." However, it is known that the position of the carbonate absorption band shifts in the presence of different cations.[84, 85] It should be possible by examining the position of this band in synthetic mixtures of calcium and magnesium carbonate to obtain additional evidence that might settle this question.

Beischer[87] has presented the absorption spectra of many types of renal calculi prepared as mineral oil mulls. Comparison with a template of the spectra of the major components is sufficient to identify these components. However, infrared methods cannot distinguish between the monohydrate and dihydrate of calcium oxalate since their spectra are identical. This distinction is made by optical analysis in visible light. The absorption near 1040 cm.$^{-1}$ in urinary calculi is due to phosphate and is not influenced by the presence of a hydroxyl group since hydroxyapatite and Ca_3PO_4 have the same spectra. However, X-ray studies have established that the calculi contain basic calcium phosphate, and the presence of this band is an indication of the presence of the hydroxyapatite. The presence of bands at 1410, 880, and 720 cm.$^{-1}$ is indicative of the presence of carbonate. The influence of cations on the 1040 cm.$^{-1}$ band of phosphate is sufficient to permit a distinction between the calcium and magnesium ammonium phosphates. The shift of this band was also noted by Lagrange and Pobeguin,[88] who used infrared and X-ray techniques to identify calculi, and emission spectroscopy to measure the concentration of such trace elements as Fe, Pb, and Si, and of Mg, Ca, and P when these were present in trace amounts. Organic constituents such as cystine and uric acid are easily identifiable by their characteristic spectra.[87] Beischer[87] was able to determine with infrared that a calculus contains a nucleus composed of cystine, that $MgNH_4PO_4$ and

hydroxyapatite increased from the center out, and that the outermost layer is composed of pure cystine. He proposed the use of KBr disks, which would permit quantitative analysis for the various constituents. However, other methods would still be necessary, for example, to determine trace amounts of other elements.

Blood and the Vascular System. Blout and Mellors[13] recorded the spectra of normal and leukemic blood smears of man. Woernley[90] gave curves for defibrinated whole blood and defibrinated serum; all these spectra appear to be very similar. The difference between the normal and pathological sera (hypersensitivity diseases) was attributed to changes in the albumin-to-globulin ratio.[91] Cook[20] recorded a spectrum of rabbit serum in KBr disk, and Potts and Wright[6] illustrated the use of water for the spectral analysis of blood. Lenormant[92, 93] studied various fractions of blood. Absorption bands at 1170 and 1093 to 1105 cm.$^{-1}$ appeared in the spectra of blood, but not in the spectra of breast tissue.[13] This latter band may be characteristic of blood spectra since it has not been found in the spectra of other tissues.[94]

Human erythrocytes[40, 95] were found to have a spectrum very similar to that of whole blood. The spectra[95] of the fowl red cells (pigeon, duck, and chicken) differed from those of mammalian cells (guinea pig, sheep, dog, and human). The band at 980 cm.$^{-1}$ in the latter spectra was missing in the spectra of fowl cells. In addition, the ratios of the absorbances at 1100 and 1550 cm.$^{-1}$ were higher for the fowl cells than for the mammalian cells. The bands at 1235, 1075, and 962 cm.$^{-1}$ in the fowl-cell spectrum were stronger than the corresponding bands in the spectra of the mammalian cells. Lenormant[9] obtained the spectrum of leukocytes suspended in D_2O in the 6 to 7 μ region. The single band at 1550 cm.$^{-1}$ in films of red cells appeared as three separate bands. This may be due to deuterization of various constituents of the cell, for example, the proteins.

A comparison of the spectra of adult aorta[90] and atheromatous aortic tissue of rabbit[96] revealed that the latter spectrum had a band at 1730 cm.$^{-1}$, which did not appear in the former. This band disappeared when the atheromatous tissue was treated with acetone and chloroform.[96] The spectrum of the residue after extraction resembled that of protein material. The lipide character of the diseased tissue was indicated by bands at 1175, 1380, 1470, 1730, and 2940 cm.$^{-1}$ that disappeared or diminished in intensity after extraction. The individual lipides were identified from their spectra after separations as cholesterol, cholesteryl esters, and phospholipides.

Schwarz *et al.*[97] studied the bone marrow of rats before and after irradiation with X rays. The bands in the 8.1 to 11.0 μ region are assigned to nucleic acid absorptions. The decrease in the absorption in this region after irradiation indicated that there had been a decrease in the nucleic acid content.

Muscle. There have been numerous studies of dried muscle,[14, 68, 101, 102] dried muscle fiber,[103] and living muscle.[10, 102, 104] The disagreement between spectra is due to differences in the methods of preparing the specimens, the type of muscle, and the species. Wood[10] observed differences in the spectra of muscles from frogs. The intensities of the bands differ and there is no band at 1031 cm.$^{-1}$ This latter is absent in the spectra of muscles from cockroach

and rabbit and some muscle preparations of turtle. However, further work is required before any correlation between the presence or absence of this band can be made with any physiological or structural factors.

The use of muscle films permits a recording of the spectrum in the whole infrared region, but does not allow an examination of living muscle. This can be done in aqueous solution. Wood[5] used Ringer's solution in both the specimen and reference cells since this solution is necessary to keep the muscle functioning. However, the water still absorbs strongly in the 3 and 6 μ regions, where the major protein absorption bands appear. The substitution of D_2O for ordinary water has been only partially successful.[10]

Muscle in different conditions of stimulation has been studied, and there is little agreement on the changes observed.[11, 101, 102, 105] Elliot[105] observed "little difference" between the spectrum of resting muscle and that of muscle shortened 25 per cent from the normal and re-extended. Dubusson *et al.*[101] reported that muscle treated with monoiodoacetic acid and then stimulated isometrically had the same spectrum as resting muscle. The spectrum of muscles contracted isotonically showed a decrease in the intensity of the band at 1570 cm.$^{-1}$ Morales and Cecchini[11] found no gross changes between the spectra of resting and isotonically contracted muscles, but did observe minor differences near 930 and 1200 cm.$^{-1}$ These different observations might be related to the methods of preparing the specimens. For example, Dubusson *et al.*[101] froze and dried the sample, but Morales and Cecchini[11] prepared films by pressing the specimens at about 3000 p.s.i. after freezing and drying. The pressure may induce alterations in the structure of the muscle constituents contributing to the infrared absorption, and this may account for the varying results obtained by different authors.[14, 101]

Elliot[105] stated that the spectrum of muscle resembles α- rather than β-keratin. Malcolm[102] confirmed this in his observations of the near infrared dichroism of live resting and dried stretched muscles. A great similarity is seen between these spectra and the spectrum of oriented α-polyalanine fiber. He concluded that "there are no grounds for supposing muscular contraction to be predominantly a β to α transition." Barer[106] reported that no infrared dichroism was observed in the 3 to 14 μ region with single muscle fibers. However, he stated that infrared dichroism may be observed only under special conditions.

The spectra of myosin[101, 104] and actomyosin[11] closely resemble the spectrum of muscle in the region 1200 to 3100 cm.$^{-1}$ The bands below 1200 cm.$^{-1}$ were attributed to adenine nucleotides or to glycogen, but a study of glycogen and adenosine triphosphate in the concentration range present in the tissue revealed that these substances do not contribute to these bands.[101] The constituents responsible for the absorption at 1030 cm.$^{-1}$ still remain to be uncovered, and perhaps extraction procedures will provide the answer.

Nervous Tissue. In general, the spectra are qualitatively identical for nervous tissue excised from different areas of the nervous system, from various species, and even when different methods of sacrificing the animals were used.[11, 12, 68, 90, 94, 103, 107, 108] However, Schwarz and his co-workers,[12, 94, 107, 108] Woernley,[90] and Barer *et al.*[103] did not report an absorption band at 1730 cm.$^{-1}$ This

is surprising because this band arises from absorptions of ester C=O, which is characteristic of lipides. This band appears on the side of a very strong band at 1654 cm.$^{-1}$, and the failure to record it may be due to the sample preparation (mashed mouse brain[90]) or to poor resolution.[12, 94, 107, 108] Schwarz and his co-workers[12, 94, 107, 108] also failed to record a band at 3050 cm.$^{-1}$, perhaps for this same reason. Coates *et al.*[109] reported slight differences in the spectra of white and gray matter of hypothalamus, using a microscope. Grenell and May[11] made suggestive correlations for the absorption bands in the tissue spectrum, based upon the spectra of brain constituents recorded in the literature. It was suggested that the spectrum of a liponucleoprotein would most nearly approximate the tissue spectrum.

Schwarz and his co-workers[94, 107] proposed a quantitative scheme to evaluate the spectra (FIGURE 1). The ratios of the absorbances at 970 and 1550 (K_1) and 1070 and 1550 cm.$^{-1}$ (K_2) were used, on the assumption that the band at 1550 cm.$^{-1}$ was due to protein. Duplicate spectra of the same tissue of different thicknesses gave almost identical K values. The bands at 1070 and 970 cm.$^{-1}$ were characteristic of spectra of nervous tissue. Variations of K values among different areas of the brain were reported, and it was stated that the measurements should only be done if the bands were clearly distinguishable. It should be noted that other substances such as lipides[11] (cephalin, and lecithin) contribute to the band at 1550 cm.$^{-1}$ and that the light scattering of the

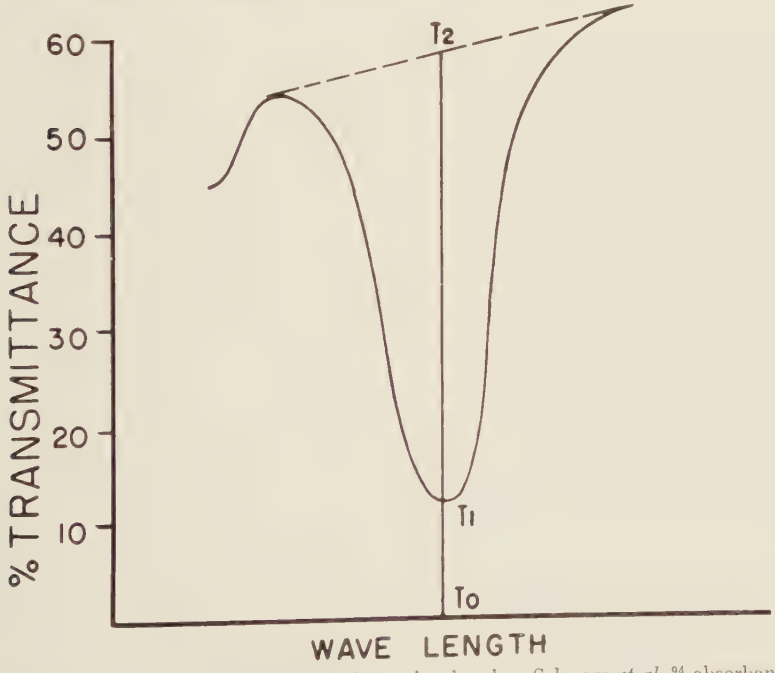


FIGURE 1. Quantitative analysis of absorption bands. Schwarz *et al.*,[94] absorbance = $-\log T_1$; Thomas *et al.*,[17] intensity = $T_2 - T_1$; and baseline method,[114] absorbance = $\log (T_2/T_1)$.

tissue sections would alter the absorbance of these bands. Thomas *et al.*[17] determined the intensity of the bands in a manner shown in FIGURE 1. To compensate for variation in film thickness, ratios were calculated between the intensities of the appropriate bands and the 1235 cm.$^{-1}$ band, since the intensity of this latter band remained relatively constant. The intensities of the bands at 1750, 1470, 1408, 1390 and 720 cm.$^{-1}$ were used in calculating the ratios. Significant variations were observed between gray and white matter. It should be noted that an examination of published curves failed to reveal the presence of a band at 1408 cm.$^{-1}$ in most instances, and in others it appeared as a weak band. Davies and Thomas[110] applied this technique to changes in the spectrum of rat spinal-cord homogenates with the age of the animal. The ratios of the intensities of 1750 1235, 1470 1235, 1390 1235, and 720 1235 cm.$^{-1}$ increased with age, but the ratio of the intensities of 1408 1235 reached a maximum and then declined. The changes occurred during the time myelin was being synthesized but, in view of the different changes of the ratios, Davies and Thomas suggested that three or four different chemical processes were being followed by this technique. The bands and their correlations as well as other correlations are shown in TABLE 1. This empirical method is based upon the constancy of the 1235 cm.$^{-1}$ band, which includes contributions from nucleic acids. The correlations observed between white and gray matter are fortuitous because the differences in the nucleic acid analyses in these areas are very small. The technique may not be applicable when a comparsion is made between the cerebral gray matter and the cerebellum, where differences in nucleic acid compositions are much greater.[111-113] Schwarz *et al.*[108] showed that the spectra of fetal brain and adult brain are different in the region between 8.1 and 11 μ. Grenell and May[11] used these quantitative techniques as well as the baseline method[114] (FIGURE 1) and found that, with their single-beam instrument, none of the methods could quantitatively distinguish between gray and white matter.

Extraction of nervous tissue with lipide solvents such as chloroform-methanol mixture reduces the intensity of many bands.[11, 105] Schwarz *et al.*[94, 107, 108] reported that the bands at 970 and 1075 cm.$^{-1}$ "completely disappeared after extraction" with chloroform-ether mixture, but a close examination of the published curves[94] (FIGURE 1) for the extraction of sections of rat cerebellum

TABLE 1

ABSORPTION BANDS USED BY DAVIES AND THOMAS*, AND CORRELATIONS

Band cm.$^{-1}$	Group vibration*	Other correlation†
1754	C=O, fatty acids, myelin	Lipides
1470	C—H bending, CH_2	Lipoprotein, nucleic acid, lipides
1408	C—H bending, CH_3	
1390	C—H bending, CH_3	Lipoprotein, nucleic acid, lipides
1235	—	Lipoprotein, nucleic acid, lipides
720	$-(CH_2)_4-$	Lipoprotein, nucleic acid, lipides

* Davies and Thomas.[110]
† Grenell and May.[11]

and white hippocampus reveals that these bands are present at extraction. Even extraction for 50 hours at 4° C.[108] or 4 days at room temperature[11] did not remove these bands from the spectrum of the residues. This may be a distinguishing characteristic of nervous tissue, since lipide solvents did remove bands from atheromatous aortic tissue.[96]

Schwarz *et al.*[94] found changes in the spectrum of nervous tissues excised from rabbits subjected to insulin shock. They noted significant changes in their K values. In three animals that were free of shock symptoms after insulin injection, sections in different areas of the brain had K values equivalent to those observed with control rabbits. No attempt was made to relate these changes to variation in chemical composition or to alteration in the structure of any tissue constituent. No data were included to indicate the physiological state of the rabbits treated with insulin.

These authors[105] used chloroform-ether to extract lipides from different areas of adult rat brain, from fetal rat-brain tissue, and from brain tissue of rats exposed to X-ray irradiation. The ratios of absorbances at 970, 1050, and 1080 to the 1550 cm.$^{-1}$ band were measured in the extracted and tissue spectra. The ratio of the values before and after extraction was taken to be the ratio of the amide-free lipide fraction to proteins of the brain. These ratios were found to vary from one area to another. It was concluded that the amount of amide-free lipides is smaller in the fetal than in the adult brain. This is in agreement with the results of Brante,[115] who showed that the lipide content is lowest in the fetal brain of humans. X-ray irradiation decreases the amide-free lipide fraction in the adult brain, but increases that fraction in the fetus.

Visceral and Other Tissues. Schwarz *et al.*[12, 94] have examined the spectra of a large number of animal tissues. They indicated that the fingerprint pattern between 9 and 11 μ shows differences between tissues. Tissues from brain, heart, liver, spleen, thymus, and lymph show great similarity in this region. However, nervous tissue has a band[11, 17, 68] at 1735 cm.$^{-1}$ not observed with these other tissues. Pancreas[90] and fat[68, 90] have the band at 1735 cm.$^{-1}$ with a different spectrum in the fingerprint region. Differences are also observed between tissues at wave lengths greater than 11 μ, so that characterizations of tissue spectra should not be limited to this region alone. The differences may be related to variations in intensity of bands and, with the development of a quantitative method, it may be possible to differentiate between tissues with similar spectra.

Schwarz *et al.*[94] studied the effect of insulin shock on the spectra of various tissues. In addition to the observations reported in the section on *Nervous Tissue*, they observed no changes in the spectra of thymus, spleen, heart, and kidney cortex, but did find changes in the spectra of liver, kidney medulla, and adrenal. They suggested that more animals of different species must be studied to verify these findings.

Blout and Mellors[13] compared the spectra of mammary fibroadenoma, mammary carcinoma, and normal breast tissue (all human). They observed an increase in the intensity of the 1075 cm.$^{-1}$ band in the neoplastic tissue. Woernley[90] found increased absorption of this band, as well as bands at 1235 and 970 cm.$^{-1}$ Ceselli and Guzzi[116] studied the variation of the spectrum of livers from

rats treated with dimethylaminoazobenzene and also observed increases in intensities of these bands. These bands were correlated with nucleic acid absorption.[90] Fraser and Chayen[37] provided additional evidence that nucleic acids are partially responsible for these absorption bands. They recorded the spectrum of ram spermatozoa before and after extraction with 0.1 *N* HCl, which removes nucleic acid. Woernley[90] concurs with the opinion of Blout and Mellors[13] that the increase in intensity of the 1075 cm.$^{-1}$ band "is due to an increased amount of nucleic acids in the rapidly proliferating and more cellular carcinoma."

Schwarz *et al.*[117] identified the characteristic band (1040 cm.$^{-1}$) of glycogen in the liver spectrum by comparison of these two spectra. This band did not appear in the liver spectrum after treatment with cold trichloroacetic acid, which is a solvent for glycogen. This band was not found in the spectrum of liver from rats that had fasted, but reappeared in the liver spectrum of animals that had been fed sucrose. A semiquantitative estimation of glycogen in liver was based upon the ratio of the absorbances at 1040 and 1550 cm.$^{-1}$ It was found that fetal rats had a greater glycogen content in the liver than did adult rats. X-ray irradiation produced no change in the glycogen content of the liver of nonfasted animals, but sucrose-fed animals showed a decrease and fasted animals an increase.

Scrocco and Benedetti[115] studied the 3 μ absorption bands of the N—H bond in collagen and reticular tissue of the kidney and lymph node. They found bands at 3328, 3308, 3312, and 3302 to 3318 cm.$^{-1}$ These latter bands varied with fetal age of embryo collagen tissue of oxen. These bands shifted to higher wave numbers after trypsin digestion.

Subcellular Structures. Benedict[45] found that the spectrum of the nuclear fraction of chicken erythrocytes is identical with the spectrum of the whole cell in the limited region examined (6 to 12 μ). However, Woernley's[90] spectra of nuclei from mouse liver and mouse tumor are different in both intensity and number of bands from the whole-cell spectra. He separated the cytoplasm of both tissues into light and heavy fractions. Differences in the spectra were observed for these fractions, but were less pronounced with tumor than with liver. De Lozé and Lenormant[113] examined the spectra of isolated mitochondria from the livers of guinea pig, rabbit, and mouse and the pancreas of dog. The mitochondria were prepared as dried films or suspended in water or D_2O. The spectrum of guinea pig liver suspended in water is not the same as that of the dried film. However, a comparison of the spectra of the water suspensions indicates species differences. It is interesting to note that the spectra of the mitochondria of liver from rabbit and guinea pig have a band at 1725 cm.$^{-1}$ but that this band does not appear in the spectrum of mitochondria of dog pancreas. Whole cells from mouse pancreas or liver give a spectrum devoid of this band.[90] Woernley's spectrum of the light fraction of mouse-liver cytoplasm shows a shoulder in this region.[90] The different spectra of pancreatic and liver mitochondria indicate differences in composition and hence some different degree of activity. Differences have been observed between the behavior of brain and liver mitochondria toward the reaction with the drug chlor-

promazine.[120] All the mitochondrial spectra are similar to the spectrum of α-protein in the 6 μ region.

The investigation of subcellular structures is of great importance to the understanding of biochemical interrelationships in the cell. These can be examined *in situ* with the infrared microscope or by studying cellular fractions isolated by biochemical methods. The microscope does not yet appear to have sufficient resolution to distinguish subcellular entities from one another. As a consequence, these studies can be carried out only after isolation of particular structures.

Summary

Infrared spectral analysis of tissues has been used to a great extent as a tool for identification of living organisms or parts thereof. Obvious complications arise, however, from the chemical complexity of living material. The problems and limitations with which the experimental biologist is constantly faced not only are present here, but are responsible for creating difficulties with which other investigators of pure physical or chemical systems need not cope. Consequently, biological infrared analysis must be carried out either on an unknown of infinite complexity, so that it becomes almost impossible to delineate individual factors, or on extracted components that no longer bear any real resemblance to the functioning living system.

Fortunately, the identification of whole tissues or organs is not a major goal of the biological infrared spectroscopist. An exception may be found in studies of bacteria. In this case, the spectral method may provide a technique that is faster than the classical methods. However, it is clear that the spectra of whole cells only will not differentiate between all species and strains. A combination of the spectra of whole cells and those of extracts may provide the necessary information for complete identification. It may be important to clarify the reasons for the absence or presence of certain bands in the spectra and their relationship to the biological activities of the different strains.

In some instances, infrared analysis has provided a means for the identification and semiquantitative estimation of cellular constituents. Since, as has been pointed out, the chemical composition of tissue is highly complex and in many instances not completely known, this procedure is useful only in special cases. Quantitative determinations on whole-tissue spectra of necessity must be based on empirical or semiempirical methods. This formidable obstacle will be overcome only when the problem of light scattering is eliminated and the contribution of all cellular chemical constituents to the spectrum can be truly evaluated. Isolation of specific chemical entities by extraction and analysis with infrared techniques is at present the most fruitful, although limited, approach to quantitative analysis.

Although infrared spectra can provide quantitative data in most biological systems, other methods such as chemical analysis, ultraviolet spectroscopy, and fluorometry are indispensable adjuncts to the completion of a total analytical method. These other techniques alone can provide quantitative analysis of chemical entities. The greatest value of infrared spectroscopy, however, is

in the determination of the molecular patterns associated with cell structure and function. Direct determinations of structure of a particular chemical species from the spectra of whole cells are complicated by the factors mentioned above.

Although changes in the functional status of cells may be reflected in altered spectra, the interpretation of the structural changes is hampered by a lack of knowledge of which chemical species has contributed to the changed bands, to the appearance of new bands, or to the disappearance of bands. The spectral shifts may afford data relative to certain molecular structures and mechanisms associated with the observed functional alterations, but cannot give positive proof of either the cell change itself or of the identification of the specific molecules involved. Extraction procedures should afford the means to isolate the particular molecular species that has altered its structure. Ultimately it becomes necessary to know what changes have occurred in the constituents of the cell without radically changing cellular organization from the *in vivo* status. Hence, the extraction procedure to be adopted should be the mildest possible, so that the structure of the molecular species is not significantly altered in the extraction. Other spectroscopic techniques, such as Raman and ultraviolet, and X-ray diffraction, provide additional information for the interpretation of chemical structure. These and infrared techniques are of great value in the study of tissues (such as those referred to as osteoid) whose major constituent is one molecular species, so that extraction procedures may not be required.

In general, then, biological infrared spectroscopy thus far is of major importance in the study of the dynamic chemical anatomy of the cell. Consequently, it appears to be of particular value in the investigation of systems in which available supplementary theory and data have shown molecular patterns and shifts to be fundamental. In nervous tissue, for example, a great deal of evidence strongly suggests that the molecular relationships in the cell membranes establish the foundation for functional activity. In fact, a number of years ago it was suggested by Pauling[121] that transmission of a nerve impulse primarily involved the shift of H-bonds. Perhaps the time is not too far distant when the investigation of such problems may be carried out by means of infrared techniques.

In most cases, however, in order to elucidate the structural alterations in molecular species, it will be necessary to combine the information gained from the study of the spectra of whole cells and of the extractants or residues after extraction. This is a long-term project and must be done with great care at each stage.

References Listed by Tissues

Plants. Nos. 23 to 37.
Microorganisms. Nos. 9, 16, 38 to 45, 47 to 57.
Insects. Nos. 36, 58 to 62.
Skin. Absorption: Nos. 60, 63 to 73, 79 to 81. Reflectance: Nos. 71 to 78.
Osteoid tissues. Nos. 82 to 89.
Blood and the vascular system. Nos. 6, 9, 13, 20, 90 to 93, 95 to 100.
Muscle. Nos. 5, 10, 14, 68, 101 to 106.
Nervous tissue. Nos. 11, 12, 17, 68, 90, 94, 103, 107 to 110.
Visceral and other tissues. Nos. 1, 12, 13, 37, 68, 90, 94, 116 to 118.
Subcellular structures. Nos. 90, 95, 119.

References

1. Clark, C. 1952. Appl. Spectroscopy. **6**(3): 14.
2. Clark, C. 1955. Physical Techniques in Biological Research. G. Oster and A. W. Pollister, Eds. **1:** 312. Academic Press. New York, N. Y.
3. Freeman, N. K. 1955. Advances in Biol. and Med. Phys. **4:** 167.
4. Sutherland, G. B. B. M. 1952. Advances in Protein Chem. **7:** 291.
5. Wood, D. L. 1950. Rev. Sci. Instr. **21:** 764.
6. Potts, W. J., Jr. & N. Wright. 1956. Anal. Chem. **28:** 1255.
7. Sternglanz, H. 1956. Appl. Spectroscopy. **10**(2): 77.
8. Blout, E. R. 1957. Ann. N. Y. Acad. Sci. **69**(1): 84.
9. Lenormant, H. 1953. Compt. rend soc. biol. **147:** 406.
10. Wood, D. L. & G. B. B. M. Sutherland. 1952. Federation Proc. **11:** 175.
11. Grenell, R. G. & L. May. 1957. J. Neurochem. In press.
12. Schwarz, H. P. 1952. Appl. Spectroscopy. **6**(4): 15.
13. Blout, E. R. & R. C. Mellors. 1949. Science. **110:** 137.
14. Morales, M. F. & L. P. Cecchini. 1951. J. Cellular Comp. Physiol. **37:** 107.
15. Blout, E. R. Personal communication.
16. Thomas, L. C. & J. E. S. Greenstreet. 1954. Spectrochim. Acta. **6:** 302.
17. Thomas, L. C., L. Austin & D. R. Davies. 1954. Spectrochim. Acta. **6:** 320.
18. Schiedt, U. & H. Reinwein. 1952. Z. Naturforsch. **7b:** 270.
19. Stimson, M. M. & M. J. O'Donnell. 1954. J. Am. Chem. Soc. **74:** 1805.
20. Cook, E. S., C. W. Kreke, E. B. Barnes & W. Motzel. 1954. Nature. **174:** 1144.
21. Stewart, J. E. 1955. Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. Anal. Chem. **27:** 318.
22. May, L. Unpublished results.
23. Schubert, W. J. & F. F. Nord. 1950. J. Am. Chem. Soc. **72:** 3835.
24. Bergmann, G., G. Huck, J. Karweil & H. Luther. 1954. Brennstoff.-Chem. **35:** 175.
25. Kratzl, K. & H. Tschamler. 1952. Monatsh. Chem. **83:** 786.
26. Tschamler, H., K. Kratzl, R. Leutner, A. Steininger & J. Kisser. 1953. Mikroskopie. **8:** 238.
27. Brauns, F. & H. Seiler. 1952. Tappi. **35:** 67.
28. De Baun, R. & F. F. Nord. 1951. J. Am. Chem. Soc. **73:** 1358.
29. Kudzin, S. F. & F. F. Nord. 1951. J. Am. Chem. Soc. **73:** 690.
30. Jones, E. J. 1949. Dissertation. Institute of Paper Chemistry. Appleton, Wis.
31. Jones, E. J. 1948. J. Am. Chem. Soc. **70:** 1984.
32. Jones, E. J. 1949. Tappi. **32:** 167.
33. Gates, D. M. & W. Tantraporn. 1952. Science. **115:** 613.
34. Swanson, C. P., C. S. Rupert & H. T. Yost, Jr. 1953. Am. J. Botany. **40:** 557.
35. Herbst, W. 1955. Z. Pflanzenkrankh. u. Pflanzenschutz. **62:** 370.
36. Stair, R. & W. W. Coblentz. 1935. J. Research Natl. Bur. Standards. **15:** 295.
37. Fraser, R. D. B. & J. Chayen. 1952. Exptl. Cell Research. **3:** 492.
38. Stevenson, H. J. R. & O. E. A. Bolduan. 1952. Science. **116:** 111.
39. Levine, S., H. J. R. Stevenson, L. A. Chambers & B. A. Kenner. 1953. J. Bacteriol. **65:** 10.
40. Simon, S. & L. R. Hedrick. 1954. Bacteriol. Proc. : 26.
41. Kabler, P. W., J. W. Riddle & B. A. Kenner. 1956. Bacteriol. Proc. : 103.
42. Levine, S., H. J. R. Stevenson, R. H. Bordner & P. R. Edwards. 1955. J. Infectious Diseases. **96:** 193.
43. Kull, F. C. & M. R. Grimm. 1954. Bacteriol. Proc. : 26.
44. Kull, F. C. & M. R. Grimm. 1956. J. Bacteriol. **71:** 342.
45. Kull, F. C. & M. R. Grimm. 1956. Bacteriol. Proc. : 105.
46. Baer, E., J. Maurukas & M. Russells. 1952. J. Am. Chem. Soc. **74:** 152.
47. Levine, S., H. J. R. Stevenson & R. H. Bordner. 1953. Science. **118:** 141.
48. Blout, E. R. & H. Lenormant. 1953. J. Opt. Soc. Am. **43:** 1093.
49. Levine, S., H. J. R. Stevenson, E. C. Tabor, R. H. Bordner & L. A. Chambers. 1953. J. Bacteriol. **66:** 664.
50. Smith, D. W., H. M. Randall, M. M. Gastambide Odier & A. L. Koevoet. 1957. Ann. N. Y. Acad. Sci. **69**(1): 145.
51. Levine, S., H. J. R. Stevenson & P. W. Kabler. 1953 Arch. Biochem. Biophys. **45:** 65.
52. Stevenson, H. J. R. & S. Levine. 1952. Science. **116:** 705.
53. Schneider, M. D. & J. McLaughlin, Jr. 1954. Bacteriol. Proc. : 78
54. Schneider, M. D. & J. McLaughlin, Jr. 1955. J. Bacteriol. **70:** 87.

55. Levi, L., B. H. Matheson & F. S. Thatcher. 1956. Science. **123:** 64.
56. Shirk, H. G. & G. A. Greathouse. 1952. Anal. Chem. **24:** 1774.
57. Astbury, W. T. & N. N. Saha. 1953. Nature. **171:** 280.
58. Coblentz, W. W. 1911. Bull. Bur. Standards. **7:** 619.
59. Coblentz, W. W. 1912. A Physical Study of the Fire Fly. Carnegie Inst. Wash. Publ. **164**.
60. Rücker, F. 1933. Pflügers Arch. ges. Physiol. **231:** 742.
61. Micks, D. W. & A. A. Benedict. 1953. Proc. Soc. Exptl. Biol. Med. **84:** 12.
62. Darmon, S. E. & K. M. Rudall. 1950. Discussions Faraday Soc. **9:** 251.
63. Krüger, P. 1929. Biol. Zentr. **49:** 65.
64. Krüger, P. 1931. Z. Morphol. Ökol. Tiere. **22:** 759.
65. Cartwright, C. H. 1930. J. Opt. Soc. Am. **20:** 81.
66. Forsythe, W. E. & F. L. Christison. 1930. J. Opt. Soc. Am. **20:** 693.
67. Bachem, A. & C. I. Reed. 1931. Am. J. Physiol. **97:** 86.
68. Lenormant, H. 1945. Compt. rend. **220:** 711.
69. Hardy, J. D. & C. Muschenheim. 1934. J. Clin. Invest. **13:** 1.
70. Hardy, J. D. & C. Muschenheim. 1936. J. Clin. Invest. **15:** 1.
71. Clark, C., R. Vinegar & J. D. Hardy. 1953. J. Opt. Soc. Am. **43:** 993.
72. Hardy, J. D., H. T. Hammel & R. Vinegar. 1955. Federation Proc. **14:** 69.
73. Hardy, J. D., H. T. Hammel & D. Murgatroyd. 1956. J. Appl. Physiol. **9:** 257.
74. Dimitroff, J. M., H. F. Kuppenheim, I. C. Graham & C. W. McKeehan. 1956. J. Appl. Physiol. **8:** 532.
75. Jacquez, J. J., J. Huss, W. McKeehan, J. M. Dimitroff & H. F. Kuppenheim. 1955. J. Appl. Physiol. **8:** 297.
76. Kuppenheim, H. F. 1954. Proc. 1st Intern. Photobiol. Congr. : 228.
77. Kuppenheim, H. F., J. M. Dimitroff, P. M. Melotti, I. C. Graham & D. W. Swanson. 1956. J. Appl. Physiol. **9:** 75.
78. Krolak, L. J. & T. P. Davis. 1955. Univ. Rochester Atomic Energy Project Rept. UR-380.
79. Alpen, E. L., C. P. Butler, S. B. Martin & A. K. Davis. 1956. J. Appl. Physiol. **8:** 399.
80. Danforth, R. 1930. Proc. Soc. Exptl. Biol. Med. **27:** 283.
81. Heer, R. R., Jr. 1952. Science. **115:** 15.
82. Caglioti, V., A. Ascenzi & M. Scrocco. 1954. Experientia. **10:** 371.
83. Caglioti, V., A. Ascenzi & M. Scrocco. 1954. Atti accad. nazl. Lincei. Rend. Classe sci. fis. mat. e nat. **16:** 180.
84. Posner, A. S. & G. Duyckaerts. 1954. Experientia. **10:** 424.
85. Underwood, A. L., T. Y. Toribara & W. F. Neuman. 1955. J. Am. Chem. Soc. **77:** 317.
86. McConnell, D. 1955. Biochim. et Biophys. Acta. **17:** 450.
87. Beischer, D. E. 1955. J. Urol. **73:** 653.
88. Lagrange, R. & T. Pobeguin. 1956. Compt. rend. **245:** 449.
89. Caglioti, V., A. Ascenzi & M. Scrocco. 1955. Arch. sci. biol. Bologna. **39:** 116.
90. Woernley, D. L. 1952. Cancer Research. **12:** 764.
91. Agnew, J. T., P. Lisan & M. J. Boyd. 1952. J. Opt. Soc. Am. **42:** 815.
92. Lenormant, H. 1950. Compt. rend. **230:** 1212.
93. Lenormant, H. 1951. Compt. rend. **232:** 397.
94. Schwarz, H. P., H. E. Riggs, C. Glick, W. Cameron, E. Beyer, B. Jaffee & L. Trombetta. 1951. Proc. Soc. Exptl. Biol. Med. **76:** 267.
95. Benedict, A. A. 1954. Exptl. Cell Research **7:** 565.
96. Ng, Y. 1955. Quoted in Freeman.[3]
97. Schwarz, H. P., H. E. Riggs, C. Glick, J. McGrath, R. Childs, E. Bew, Jr. & F. Stone. 1953. J. Phila. Gen. Hosp. **4:** 165.
98. Blout, E. R., G. R. Bird & D. S. Grey. 1950. J. Opt. Soc. Am. **40:** 304.
99. Freeman, N. K. 1957. Ann. N. Y. Acad. Sci. **69**(1): 131.
100. Kramer, K., J. O. Elam, G. A. Saxton, W. N. Elam, Jr. & D. Hoeb. 1951. Am. J. Physiol. **165:** 229.
101. Dubusson, M., J. Lecomte & A. M. Monnier. 1942. Arch. intern. physiol. **52:** 408.
102. Malcolm, B. R. 1954. Symposia Soc. Exptl. Biol. No. 9. Fibrous Proteins and their Biol. Significance. : 265.
103. Barer, R., A. R. H. Cole & H. W. Thompson. 1949. Nature. **163:** 198.
104. Wood, D. L. 1951. Science. **114:** 36.
105. Elliot, A. 1952. Proc. Roy. Soc. London. **B139:** 526.
106. Barer, R. 1950. Proc. Roy. Soc. London. **B137:** 80.

107. Schwarz, H. P., H. E. Riggs & C. F. Glick. 1951. Trans. Am. Neurol. Assoc. **76:** 90.
108. Schwarz, H. P., H. E. Riggs, C. Glick, J. McGrath, W. Cameron, E. Beyer, E. Bew, Jr. & R. Childs. 1952. Proc. Soc. Exptl. Biol. Med. **80:** 467.
109. Coates, V. J., A. Offner & E. H. Siegler, Jr. 1953. J. Opt. Soc. Am. **43:** 984.
110. Davies, D. R. & L. C. Thomas. 1955. Biochemistry of the Developing Nervous System. H. Waelsch, Ed. : 170. Academic Press. New York, N. Y.
111. Grenell, R. G. & L. May. Unpublished results.
112. May, L. & R. G. Grenell. 1955. Meeting-in-Miniature. Maryland Section, Am. Chem. Soc. Baltimore, Md.
113. Palladin, A. V. 1955. Biochemistry of the Developing Nervous System. H. Waelsch, Ed. : 177. Academic Press. New York, N. Y.
114. Wright, N. 1941. Ind. Eng. Chem. **13:** 1.
115. Brante, G. 1949. Acta Physiol Scand. **18:** Suppl. 63.
116. Ceselli, C. A. & M. L. Guzzi. 1950. Tumori. **40:** 118.
117. Schwarz, H. P., H. E. Riggs, C. Glick, J. McGrath, R. Childs, E. Bew, Jr. & F. Stone. 1954. Proc. Soc. Exptl. Biol. Med. **85:** 96.
118. Scrocco, M. & E. L. Benedetti. 1955. Riv. istochim. norm. e patol. **1:** 339.
119. De Lozé, C. & H. Lenormant. 1955. J. Physiol. Paris. **47:** 231.
120. Berger, M., H. J. Strecker & H. W. Waelsch. 1956. Nature. **177:** 1234.
121. Pauling, L. 1945. The Nature of the Chemical Bond : 431. Cornell Univ. Press. Ithaca, N. Y.

THE APPLICATION OF INFRARED METHODS TO PHARMACEUTICAL ANALYSIS

By Jonas Carol

Department of Health, Education, and Welfare, Food and Drug Administration, Washington, D. C.

The analysis of pharmaceutical preparations by infrared spectrometry offers many advantages unavailable to conventional methods. Despite their obvious utility, however, infrared procedures were seldom employed for drug analysis in the past. This neglect was due largely to the high cost of the equipment required, and to technical difficulties encountered in the preparation of samples. Within the las few years, new techniques have overcome many of these limitations, and infrared spectrometric procedures for drugs have been published in increasing numbers.[1-5] Today, the routine infrared analysis of pharmaceuticals in control laboratories has become an established practice. The laboratory of the American Medical Association now includes an infrared spectrum, under tests for identity, in its monographs on new drugs. It is anticipated that similar spectra will eventually be included in the monographs of the official compendia for pharmaceuticals—the *Pharmacopeia of the United States* and *The National Formulary*.

The simplest and most accurate assays depend upon absorbance measurements on solutions of definite concentration in cells of known or compensated thickness. Analysis of single or multicomponent systems in which direct absorbance or baseline absorbance measurements are used presents few difficulties if a suitable solvent is available.*

The powerful alkaloid atropine is readily soluble in carbon disulfide, and it may be separated from the common tablet excipients by extraction with carbon disulfide. A simple extraction-spectrophotometric procedure has been devised for the determination of atropine sulfate in hypodermic tablets.[6] The identity of the extracted alkaloid is established by comparing its absorption spectrum in the region between 2 and 15 μ with the spectrum of pure atropine in carbon disulfide solution. The prominent absorption maximum at 1035 $cm.^{-1}$ (9.66 μ) is measured for quantitative analysis.

Several manufacturers' lots of atropine sulfate tablets were analyzed collaboratively by the proposed spectrophotometric procedure. The results were in close agreement with values obtained by application of the U.S.P. assay method. In the official method, total alkaloids are extracted and titrated with standard acid. The infrared spectrophotometric method requires far less time and effort, and it is far more reliable, since it provides a positive identification of the alkaloid sought.

A proposed infrared spectrophotometric assay for nitroglycerin in tablets[7] offers similar advantages of convenience and specificity. In this procedure, again, the active substance is separated from excipient materials by extraction

* Carbon tetrachloride and carbon disulfide are the only two solvents that are appreciably transparent (in thickness up to 1.0 mm.) from 2 to 15 μ. A number of solvents have "windows" in limited regions of the spectrum, or may be used in thin cells (0.1 mm. or less).

with carbon disulfide, and its concentration in the solution obtained is calculated from baseline absorbance measurements. Of the several distinctive maxima available, the absorption band at 1265 cm.$^{-1}$ (7.90 μ), characteristic of nitrate esters, is employed for the determination of nitroglycerin. Several organic polyhydroxy nitrates exhibit similar infrared absorption spectra, but they are soluble only with difficulty in carbon disulfide. Furthermore, the proposed method allows the detection of decomposition products. The analytical results for five commercial samples of nitroglycerin tablets agreed with the manufacturers' declarations and with U.S.P. assay values. In the official method, ether-soluble nitrogenous substances are reduced to ammonia, titrated, and calculated indiscriminately as nitroglycerin.

Futher examples of such simple, direct spectrophotometric procedures include the determination of sodium *N*-lauroyl sarcosinate in tooth paste,[8] of phenobarbital in tablets,[9] and of testosterone and progesterone in suspensions.[10]

Many pharmaceutical substances are not sufficiently soluble in organic solvents to produce mixtures suitable for accurate infrared measurements. In these instances, the compounds sought may be converted to soluble derivatives, or they may be incorporated in films,[11] mulls,[12] emulsions,[13] or potassium bromide disks.[14, 15] Some of these procedures may be employed for quantitative as well as qualitative analysis, if sufficient care and attention to pertinent details are exercised.

A method for the infrared spectrophotometric determination of penicillin G in the presence of penicillin F, K, or dihydro-F by a mull procedure has been described by Garlock and Grove.[16] Their method is designed to eliminate the use of the internal standard originally employed by Barnes *et al.*[12] An accurately weighed amount of mull (one part penicillin to three parts mineral oil) is placed between salt plates separated by a brass spacer 0.0024 in. thick. The properly prepared assembly contains no air bubbles and is nowhere in contact with the spacer. Penicillin G is estimated by means of a baseline absorbance measurement at the 14.2 μ band. The same technique serves for the determination of penicillin O at 990 cm.$^{-1}$ (10.10 μ). In each case, the results of analyses of commercial samples compare favorably with those obtained by other procedures.

The advent of the potassium bromide disk technique makes the infrared analysis of insoluble substances practicable. Excellent spectra are obtained with samples of 1 mg. or less of a drug. Since potassium bromide is uniformly transparent out to 500 cm.$^{-1}$, background absorption is virtually eliminated. When particle size is reduced to 0.1 μ or less, scattered light at short wave lengths is negligible, and absorptions conform to Beer's law.

The infrared analysis of ethisterone (ethinyl testosterone) in tablets[17] is typical of methods using the disk technique. The absorption spectrum obtained with a 2 mg. sample in a 200 mg. potassium bromide disk has many well-defined peaks, and the band at 2080 cm.$^{-1}$ (4.80 μ) due to the ethinyl group at C-17, although weak, is extremely significant in identifying the steroid. Analyses of commercial samples by baseline absorbance measurements near 1055 cm.$^{-1}$ (9.48 μ) check U.S.P. assays closely.

The infrared absorption spectrum of every pharmaceutical substance is both

complex and unique. These properties permit the analysis of multicomponent mixtures without a preliminary separation. Washburn and Krueger[18] have devised a spectrometric assay procedure for mixtures of aspirin, phenacetin, and caffeine. The drugs are extracted from the powdered preparation with chloroform, and standard solutions of each of the substances in chloroform are prepared. Absorbances of these solutions are measured at 1750, 1650, and 1510 cm.$^{-1}$ (5.70, 6.05, and 6.63 μ) in 0.1 mm. cells, relative to chloroform as a blank. Concentrations of aspirin, phenacetin, and caffeine in the unknown mixture are then calculated by means of determinants. The procedure was subjected to an intensive collaborative study in sixteen laboratories, representing a significant segment of the pharmaceutical industry. Collaborative results were excellent, and the infrared procedure has been recommended for adoption as a routine assay for this preparation.

In the assay of preparations containing natural estrogenic substances, it is usually necessary to determine the concentrations of biologically potent steroids in mixtures composed of many closely related substances. Pregnant mare's urine, the chief commercial source of such mixtures, contains the ketosteroids estrone, equilin, and equilenin, and the corresponding diol pairs epimeric at C-17: the estradiols, dihydroequilins, and dihydroequilenins. To complicate matters further, certain artifacts may be formed during the manufacturing process.[19] Qualitatively, the problem is similar to that investigated by Jones, Dobriner, and their co-workers[20] in their infrared study on the structures of steroid metabolites.

Furchgott, Rosenkrantz, and Shorr[11] first published the spectra of the estrogenic steroids and noted qualitative differences in their infrared absorptions. A set of quantitative spectrophotometric procedures has been devised for the estimation of the most potent steroids in mixed estrogenic substances, estrone, equilin, and estradiol-17β (*cis*). The phenolic ketosteroids are first separated from nonketonic substances by means of Girard's reagents, and they are then converted to their benzenesulfonyl esters. Unlike the parent phenols, the ester derivatives are readily soluble in carbon disulfide. The assay procedure based upon absorbance measurements at 956 cm.$^{-1}$ (equilenin), 920 cm.$^{-1}$ (estrone), and 913 cm.$^{-1}$ (equilin) gave good recoveries when tested on twenty known mixtures of ketosteroids. Analysis of natural ketosteroid fractions yields values in close agreement with those obtained by differential colorimetry and bioassay.[21]

The determination of estradiol-17β is facilitated by the occurrence of five consecutive characteristic maxima between 10.10 and 11 μ in the absorption spectrum of its benzenesulfonate. A baseline technique is employed to measure the quantity of estradiol-17β present in mixtures containing relatively large proportions of estradiol-17α and other nonketonic steroids.[22]

Every laboratory engaged in pharmaceutical analysis is frequently confronted with the problem of identifying the active principle in a single capsule or tablet. For this type of work infrared spectrophotometry is invaluable. A barbiturate, an antihistaminic, or an alkaloid may be characterized unequivocally be means of its infrared spectrum. It is not possible, of course, to prove the identity of a substance without the spectrum of the authentic compound

for comparison. However, even in the absence of standards, important structural details may be surmised from the characteristic absorption bands due to specific atomic groupings in the molecule.

In the laboratories of regulatory agencies, it is sometimes necessary to establish the source of a pharmaceutical preparation. When authentic batch material is available, infrared analysis often provides revealing data for comparing the unknown and the known samples. Because of its graphic, documentary nature the infrared spectral chart constitutes persuasive evidence in judicial actions. It is anticipated that infrared spectrophotometry will find increasing use as a forensic as well as an analytical tool in the pharmaceutical field.

References

1. Gore, R. C. 1950. Infrared spectroscopy. Anal. Chem. **22:** 7.
2. Gore, R. C. 1951. Infrared spectroscopy. Anal. Chem. **23:** 7.
3. Gore, R. C. 1952. Infrared spectroscopy. Anal. Chem. **24:** 8.
4. Gore, R. C. 1954. Infrared spectroscopy. Anal. Chem. **26:** 11.
5. Känback, T. 1956. Recent advances in pharmaceutical analysis. Infrared spectroscopy. J. Pharm. and Pharmacol. **8:** 225.
6. Carol, J. 1954. Report on analysis of drugs by infrared spectrophotometry. J. Assoc. Offic. Agr. Chemists. **37:** 692.
7. Schwartzman, G. 1956. Analysis of nitroglycerin tablets by infrared spectrophotometry. J. Assoc. Offic. Agr. Chemists, **39:** 254.
8. Carol, J. 1955. Report on infrared spectrophotometric methods for drugs. J. Assoc. Offic. Agr. Chemists. **38:** 638.
9. Report to the Combined Pharmaceutical Contact Committee. 1956. Am. Drug Mfrs. Assoc. & Am. Pharm. Mfrs. Assoc. Washington, D. C.
10. Carol, J. 1953. The chromatographic separation of progesterone and testosterone. J. Assoc. Offic. Agr. Chemists. **36**: 1001.
11. Furchgott, R. F., H. Rosenkrantz & E. Shorr. 1946. Infra-red absorption spectra of steroids. J. Biol. Chem. **164:** 621.
12. Barnes, R. B., R. C. Gore, E. F. Williams, S. G. Linsley & E. M. Petersen. 1947. Infrared analysis of crystalline penicillins. Anal. Chem. **19:** 620.
13. Dolinsky, M. 1951. Technique for infrared analysis of solids insoluble in nonpolar solvents. J. Assoc. Offic. Agr. Chemists. **34:** 748.
14. Stimson, M. M. & M. J. O'Donnell. 1952. The infrared and ultraviolet absorption spectra of cytosine and isocytosine in solid state. J. Am. Chem. Soc. **74:** 1805.
15. Schiedt, U. & H. Reinwein. 1952. The infrared spectroscopy of amino acids. I. A new preparation technique for the infrared spectroscopy of amino acids and other polar compounds. Z. Naturforsch. **7b:** 66.
16. Garlock, E. A. & D. C. Grove. 1948. The quantitative determination of crystalline penicillin G by infrared analysis. J. Am. Pharm. Assoc. Sci. Ed. **37:** 409.
17. Carol, J. 1957. Report on analysis of drugs by infrared spectrophotometry. J. Assoc. Offic. Agr. Chemists. In press.
18. Washburn, W. H. & E. O. Krueger. 1949. Infrared determination of aspirin, phenacetin and caffeine. J. Am. Pharm. Assoc. Sci. Ed. **38:** 623.
19. Grant, G. A. & D. Beall. 1950. Studies on estrogen conjugates. *In* Recent Progress in Hormone Research. **5:** 307. Academic Press. New York, N. Y.
20. Jones, R. N. & K. Dobriner. 1949. Infrared spectrometry applied to steroid structure and metabolism. *In* Vitamins and Hormones. **7:** 294. Academic Press. New York, N. Y.
21. Carol, J., J. C. Molitor & E. O. Haenni. 1948. The determination of estrone, equilin and equilenin by infrared spectrophotometry. J. Am. Pharm. Assoc. Sci. Ed. **37:** 173.
22. Carol, J. 1950. The determination of alpha estradiol and other estrogenic diols by infrared spectrophotometry. J. Am. Pharm. Assoc. Sci. Ed. **39:** 425.

INFRARED MICROSPECTROSCOPY IN BIOLOGICAL RESEARCH

By Darwin L. Wood*
University of Michigan, Ann Arbor, Mich.

Introduction

At present, infrared microspectrophotometry can be defined as the study of infrared absorption intensities for samples smaller than the mechanical slit of the spectrometer. This definition arises from the fact that the smallest sample that can be studied by a spectrophotometer without auxiliary optics is that which just covers the entrance or exit slit of the monochromator. Ordinarily the slit[1] will be no shorter than 10 mm., and may vary from 0.01 mm. to 1 mm. in width, depending on the particular region of the spectrum under consideration. If an appropriate sample thickness is about 0.025 mm., then, with no occlusion of the radiation beam incident on the slit, the minimum sample volume would be 2.5×10^{-4} cm.^{3}, or a quarter of a milligram for substances of unit density. This is, therefore, the maximum sample size for microspectrophotometry, or the minimum for macrospectrophotometry, according to the present definition. For various reasons this minimum is not reached in actual practice, but the limit may be set in this arbitrary way for the present discussion.

For smaller samples a microilluminator of some sort must be used. The most elementary microilluminator consists[2] of a pair of infrared-transmitting lenses, one producing a reduced image of the source in the sampling space, and the other restoring the radiation to its former path with an image of the source on the entrance slit. In the original work with this device, the reduction in the minimum sample size was about 3 times, and the final minimum sample size was about 0.75×6 mm., since the slit was rather larger than 1×10 mm. Application of the arrangement to any spectrometer may reduce the sample size by a similar factor, and for the case under consideration would give a minimum of 0.33×3.3 mm., or 2.5×10^{-6} cm.^{3}

One drawback of such a system arises from the errors of the refracting elements involved, especially the chromatic aberrations. The wave-length range usually investigated covers the wide interval from $2.5\,\mu$ to $15\,\mu$, a factor of 6 in wave length compared to the factor of 2 at most required to cover the visible spectrum. Thus, chromatic aberration may be considerable, especially since not many transparent materials are available for the construction of compound lenses for the infrared. The fairly large aperture required of such a system also causes the aberrations other than chromatic to be considerable for simple lenses.

The solution to many of the optical problems connected with microilluminator design has come with the development of totally reflecting micro-optics.[3-5] In fact, the first report of infrared microspectroscopy as such[6] came shortly after the description of the Schwarzschild-type totally reflecting microscope objectives

* Present address: Bell Telephone Laboratories, Murray Hill, N. J.

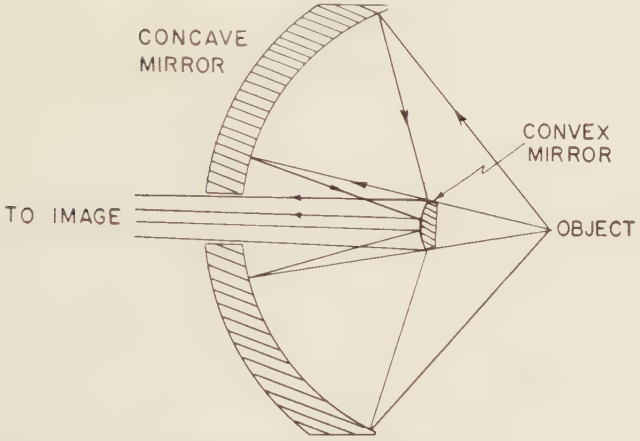


FIGURE 1. Schwarzschild-type totally reflecting microscope objective.

by Burch.[3] At the present time most infrared microspectrometers use this type of optics for the microilluminator.

Microspectrometer Optics

The reflecting microscope objective may consist of two mirror surfaces, either aspherical[3] or spherical,[5] one convex and one concave, arranged as shown in FIGURE 1. Radiation from the object is reflected from the larger concave mirror onto the small convex mirror, and then passes through the hole cut in the larger concave mirror to the image of the object. The small convex mirror obscures some of the incident radiation, and the use of aspherical mirrors is favored by some workers in order to minimize the resultant loss of energy. In a microspectrometer two of these units are arranged like an ordinary microscope, with one unit for the condenser and one for the objective, as shown in FIGURE 2. Radiation from the exit slit of the monochromator is collected by the condenser unit and is brought to a focus on the sample, forming an image of the slit at the sample. The radiation passing through the sample is then collected by the objective and passed on to the detecting system. There usually is an arrangement for visual observation of the object and a masking device in the focal plane of the visual system so that the appropriate sample areas may be selected.

It is sometimes convenient to have the microilluminator located in the undispersed radiation instead of in the exit beam from the monochromator. There is some objection to this arrangement because of the heating effect of the undispersed radiation when it is concentrated to a very small area at the sample. However, Cole and Jones[7] have made quantitative estimates of the heating effect and have concluded that the temperature rise in the sample can be kept under control. They found that an unsupported crystal with a melting point of about 70° C. did not melt at the focus of the beam, although crystals melting somewhat below this temperature did melt. If supported

by a single NaCl plate, a crystal was stable if its melting point was above approximately 40° C. When a crystal of melting point near 30° was supported between two NaCl plates, melting was not observed. Thus, the thermal conductivity of the supporting medium plays a large part in the temperature rise at the sample.

Microspectrometer Sample Sizes

In the visible region of the spectrum, absorption microspectrophotometry has been used since an early date. In this region the minimum sample size approaches that of the smallest resolvable object, and such an object would have a linear dimension equal to 0.6 λ N.A. where λ is the wave length of the light, and N.A. is the numerical aperture of the microscope objective viewing the sample. This is less than $^1/_2$ μ for visible light. In the infrared region such desirably small samples are impossible to obtain for several reasons.

First of all, the wave length is larger in the infrared, since the most interesting region lies between 2.5 and 15 μ. The diameter of the smallest object distinguishable from its neighbor at a wave length of 15 μ will be about 6 μ, assum-

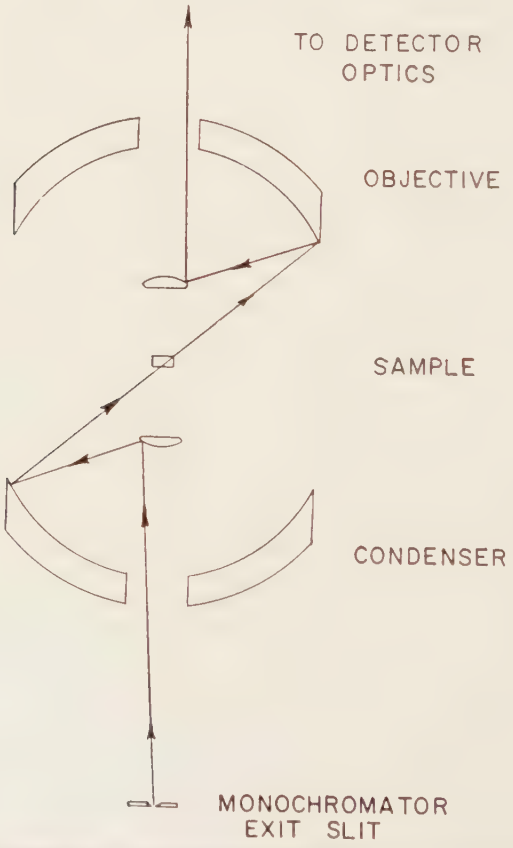


FIGURE 2. Arrangement of a microilluminator for infrared microspectrophotometry.

ing the microscope objective to have N.A. = 1.5. This is about 30 times the diameter of the minimum resolvable limit in the visible. This limit to sample size is imposed by diffraction and will be referred to as the diffraction limit.

The second factor limiting the minimum sample size in the infrared arises from the limited intensity of available sources and the limited sensitivity of available detectors. The limited amount of energy in a given spectral range for a given monochromator focal ratio sets the limit for the slit width, and this in turn sets the sample size limit in the following way.

The maximum solid angle of radiant energy that the monochromator can use is that which just fills the collimator mirror. The numerical aperture of the monochromator $(N.A.)_s$ must be related to the numerical aperture of the microilluminator $(N.A.)_m$ by the relation

$$\frac{(N.A.)_m}{(N.A.)_s} = M$$

where M is the magnification of the sample on the entrance slit of the monochromator. If the monochromator has $(N.A.)_s = 0.1$ and the microilluminator has N.A. < 1.0, then the maximum useful magnification is 10 times and the sample must have dimensions equal to $^1/_{10}$ those of the slit in order to realize the full spectral resolving power of the monochromator. Again using the maximum slit dimensions of 1 mm. × 10 mm., the minimum microsample size is 0.1 mm. × 1 mm., one order of magnitude of reduction in linear sample dimension, or two orders in cross-sectional area.

There is, however, a great disparity between this energy limit of the whole microspectrophotometer and the diffraction limit of the microilluminator. This disparity can be reduced by masking the sample and thus sacrificing energy at the detector and subsequently the spectral resolving power or response time. Alternatively, one can reduce the disparity by using sources or detectors that are less convenient or cover a smaller spectral range.

In many cases the spectra of solids or liquids have infrared absorption bands that are wide compared with the available spectral slit width. Thus, relatively little loss of detail in the spectrum will result when the sample size is reduced by masking and the energy reaching the detector is decreased. The sacrifice of spectral resolving power to reduce the sample size often can be tolerated.

If the temperature of the source is raised, the energy output in a given spectral interval will increase, and this may be used to pay for a decrease in sample size. The laboratory source having the highest energy output so far described is the carbon arc,[9] which gives an output about four times that of the conventional Nernst filament or silicon carbide Globar. Other sources such as the tungsten glower[10] and the zirconium concentrated arc[11] have greater output than the Nernst filament and Globar, although not as much as the carbon arc.

There are several detectors that have considerably higher sensitivity than the conventional thermocouple and therefore could be used to pay for a reduction in sample size. The most important of these are the photoconductors such as lead sulfide[12] or lead telluride.[13] The sensitivity of this type of detector

is as much as two orders of magnitude greater than that of a thermocouple, but its spectral range is much smaller. Thus, at the present time only the hydrogenic stretching frequencies can be reached with the photoconducting detectors, and the much more revealing absorption bands in the fingerprint region are inaccessible.

The actual limits of sample size to be expected when these innovations are used depend to a certain extent on the shape of the object to be observed; that is, fibers are better suited for microspectroscopy than spherical or circular objects because of the shape of the monochromator slits. In the case of fibers, when the sampling area is masked at the expense of spectral resolving power, it has been found[14] that spectra recorded from fibers 17 μ in diameter and 650 μ long are so sufficiently well resolved that detailed studies of the fibers can be made over the whole wave-length range from 2.5 μ to 15 μ. This represents the recording of useful spectra from about 10^{-7} gm. of material. If the carbon arc source were used instead of the standard source, this could be reduced by a factor of 2, and a small amount of further reduction could be obtained if the signal-to-noise ratio of the detecting system were increased by reducing its band pass, although this would require longer recording times. The use of a photoconducting detector and the carbon arc source would allow spectra in the hydrogenic stretching region (near 3 μ) to be recorded for fibers of diameters equal to the diffraction limit at those wave lengths.

For samples whose geometry does not resemble that of the monochromator slits, the minimum sample size must be obtained effectively by reducing the slit length. Coates, Offner, and Siegler[11] give a tabular guide to minimum sample area in which they assume a sample length of 100 μ. For sample widths ranging from 6 μ to 100 μ, this yields quite reasonable spectral slit widths and operating conditions between the wave lengths of 2 μ and 14 μ. Thus, when a sample is to be run throughout this range a well-resolved spectrum will result from a sample 100 μ square, or 2.5×10^{-7} cm.^{3} in volume, if a proper thickness of 25 μ is assumed. With a carbon arc source this can be reduced by a factor of 4 in area, giving 50 $\mu \times 50$ μ, and a sample volume near 6×10^{-8} cm.^{3} Although this work has not been published, at least one microspectrometer using a carbon arc source and conventional detector has been capable of measuring useful spectra from samples 30 μ in diameter. For convenience the relative sample dimensions discussed so far are summarized in FIGURE 3.

Samples of 30 μ diameter represent the closest experimental approach to the diffraction limit for this type of specimen and, although the method involves somewhat inconvenient apparatus, this size is a quite practical limit. It is unfortunate that so many tissue cells are smaller than 30 μ and therefore will not be amenable to individual study in the infrared. Large cells and tissues are, however, promising material for microspectroscopy.

The Effects of Convergence in Microspectrophotometry

Because of the large numerical aperture of the microilluminator system of a microspectrophotometer the object is viewed with highly convergent radiation.

This may cause some difficulties in the interpretation of data since it is usually assumed that the radiation traversing the sample is parallel.

The first effect of convergence is the change in effective thickness of the sample when viewed with the microilluminator. The central ray of the cone of radiation traverses the true thickness t of the sample. The marginal ray of the cone, however, traverses a thickness $t \cos\theta$ where θ is the angle between the cone axis and the marginal ray. This means that the effective thickness that would give the true extinction coefficient in the Beer-Lambert law is something between t and $t \cos\theta$. Blout, Bird, and Grey[5] have calculated the magnitude of this effect and give graphical data from which the appropriate correction may be made. It should be noted that in unfavorable circumstances this effect could adversely influence the contrast in a spectrum in the following way. The central bundle of rays passes through a smaller sample thickness and suffers less absorption than the marginal rays. If the marginal rays for large numerical apertures suffer nearly complete absorption at the peak of an absorption band, the higher transmission of the axial rays will apparently reduce the absorption maximum. Likewise, the minimum of absorption from the central ray bundle will be raised by the absorption of the marginal rays. Thus, a decrease in the disparity between maxima and minima in the spectrum will be observed and reduced contrast will result. This is similar to the situation observed with a strongly absorbing sample having holes through which unabsorbed radiation may pass. The contrast in such a sample is low. Although it has not been evaluated quantitatively, this effect for convergent radiation is probably small.

The second effect of convergence arises in oriented samples where dichroic ratios are being investigated. Here the absorption of marginal rays for plane-polarized radiation will vary with azimuth as well as with elevation. An oblique ray incident in the plane containing an absorbing transition moment (FIGURE 4a) will be absorbed less than an oblique ray incident in a plane per-

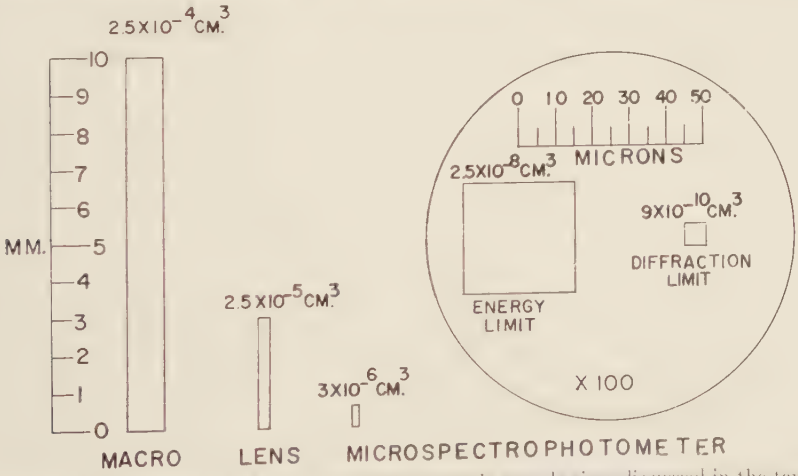


FIGURE 3. Summary of infrared spectrophotometric sample sizes discussed in the text.

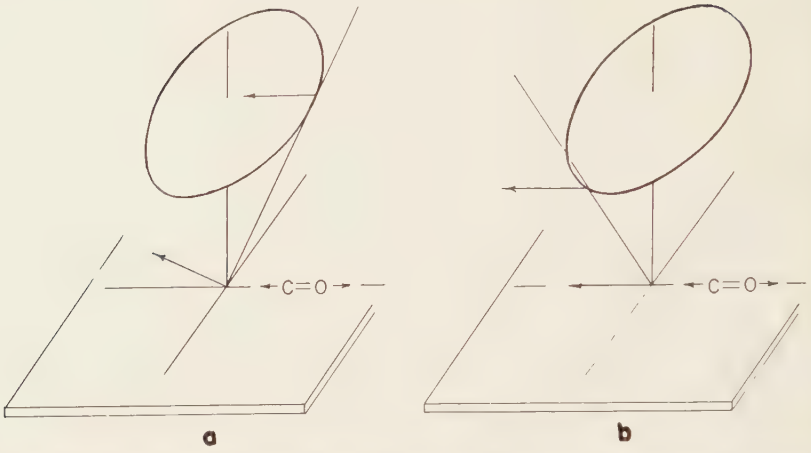


FIGURE 4. Absorption of oblique rays in convergent radiation: (a) azimuth showing decreased absorption; (b) azimuth showing absorption equal to that for normal incidence.

pendicular to the absorbing moment (FIGURE 4b). This is because in the former case the electric vector is not parallel to the moment while, in the latter case, it is parallel. Thus, the intensities for convergent radiation will differ somewhat from those observed in parallel radiation, and erroneous dichroic ratios will result. This effect has been evaluated by Fraser,[15] who has found that a small correction is sufficient.

The third effect of convergence is important only in single-crystal studies where there is a transition moment in the crystal parallel to the axis of the convergent radiation in the microilluminator. In this case the absorption is forbidden for parallel radiation, as indicated in FIGURE 5, but the marginal rays of the convergent radiation strike the sample obliquely and are absorbed. Thus, absorption bands normally forbidden for the particular crystal section will be observed in the spectrum recorded with convergent radiation. This effect may have considerable magnitude, although this type of sample will be relatively rare in biology.

In summary, one can say that the effects of convergence are not likely to be especially important in biological work but their existence should be kept in mind.

Applications to Biological Problems

In most investigations of biological problems that have involved infrared microspectrophotometry the technique has been applied to chemical systems for which only small quantities of sample were available. An intact cell is a complex system in which the more interesting components are often present in concentrations far below the modest threshold of infrared absorption. Moreover, because of their small size, it is unlikely that studies of cell parts will yield the sort of useful infromation that ultraviolet microspectrophotometry has given in the case of the cell nucleus.[16]

One favorable example, however, where useful information has been ob-

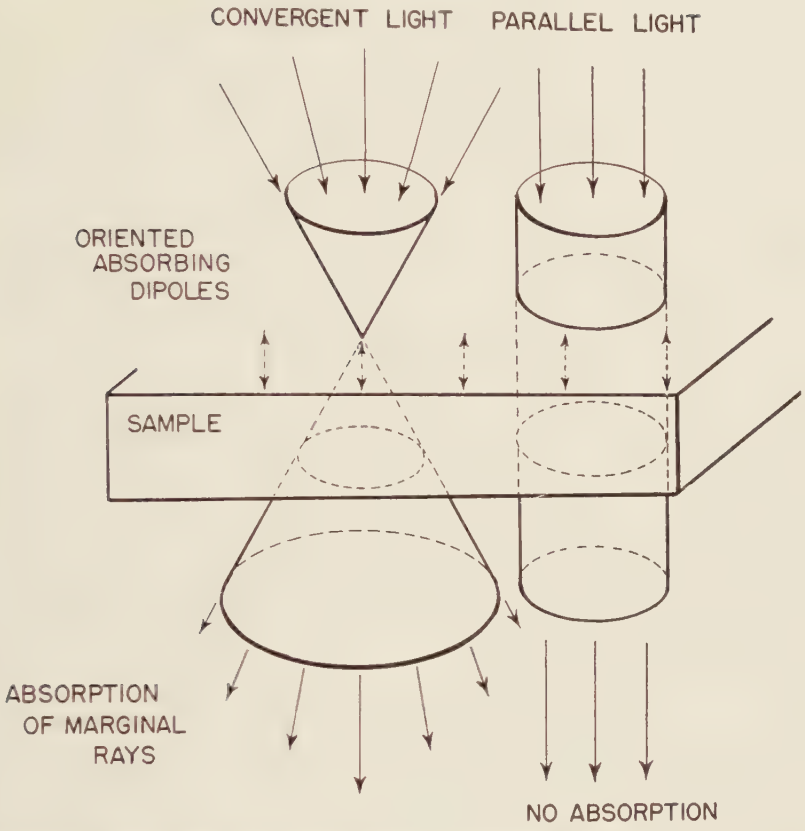


FIGURE 5. Absorption by a single crystal in convergent radiation for the case where this absorption would be forbidden in parallel radiation.

tained from single intact cells is the case of muscle. Since the individual cells are in the form of elongated fibers their geometry is well suited for microspectrophotometry, and spectra of single cells have been reported in the literature.[17] The spectra of the cells strongly resemble those of the principal protein components, myosin and actomyosin, although there are differences in the spectra of cells from different muscles in the same animal and from the same muscle in different species. These differences have never been explained. The fact that dichroism is not observed in the microspectra of intact muscle cells suggests that the contractile mechanism is constructed on a level higher than that of the elementary polypeptide chain. It also implies that the contractile process involves a more complex mechanism than the simple folding of a polypeptide chain.

Biochemical infrared microspectrophotometry has been mainly concerned with the identification of minute quantities of compounds extracted from natural systems. One such investigation[18] involved the identification from ox spleen of propionylcholine which, like acetylcholine, is an active mediator

of excitation across the neuromyelar junction. In the whole study[19] a total of about 300 μg. of the propionyl ester was available. Because of the extreme sensitivity of bioassay methods this quantity was sufficient for a large number of biological experiments, but the whole quantity was not enough for one infrared macrospectrum. The microspectrum, however, was easily recorded from about 200 μg. and the successful identification of the natural extract was easily made.

The minimum sample size in this type of work is determined by the handling procedures rather than by the limitations of the microspectrometer itself. Microgram quantities are usually handled in solution and, in many cases, the solution itself will be satisfactory for a spectrum. The sample, however, must be confined in some sort of cell. Cole and Jones[7] have described a liquid cell that 3×10^{-3} cm.^{3} of solution will fill and have recorded good spectra from 10 μg. of solute in this cell. According to their estimate, only 9×10^{-4} cm.^{3} of solution actually was in the radiation beam, and an appropriate reduction in the cell size to 9×10^{-4} cm.^{3} would have given the same spectra from about 2.5 μg. of solute.

Blout[20] has developed another technique for liquids, in which the sample is contained in a short length of silver chloride capillary tubing having a bore about 100 μ in diameter. The solutions or the pure liquids can be taken up directly in the capillary and the spectrum run without a sample transfer step. With this technique, spectra of a few micrograms of solute in a solution have been obtained.

The Future of Infrared Microspectrophotometry

What has been said thus far about infrared microspectrophotometry deals with apparatus and techniques already described in the literature, and with equipment commercially available and within the financial resources of the majority of research laboratories. The future developments that may come to pass are, of course, only a matter of speculation at this time, although some possibilities may be pointed out.

The type of information that makes infrared spectrophotometry interesting is the specific absorption intensity as a function of wave length or frequency. It is mainly the absorption in the region from 4000 to 650 cm.$^{-1}$ that is of value, although some information is also available[21, 22] in the region from 6500 to 4000 cm.$^{-1}$. Thus, the use of the infrared image converter tube as an adjunct to microscopy[23] seems unlikely to yield pertinent information since its useful range is between the visible and about 7700 cm.$^{-1}$. Photographic plates are also limited to this range outside the more useful infrared wave lengths.

There are, however, two new developments that show promise in rendering an infrared microscope image visible. These are the Evaporagraph[24] and the photographic scanning radiometer.[25] Although based on widely different principles, both of these devices produce photographs, the density or color of which depends on the intensity of the infrared radiation of particular wave lengths present in the image of an object.

Suppose an infrared image of some tissue section, for example, is formed at the focal plane of one of these devices by a reflecting microscope objective.

The blackening of the thermal photomicrograph image would depend on the infrared transmittance of the section. Thus the total absorption of the section for some wave-length interval isolated by a filter could be recorded. Even more interesting information might be obtained by combining thermal photomicrography with nondispersive spectrophotometry.[26] For example, in the scanning system of thermal photography the detector can be sensitized for a specific material such as protein by masking the surface with a protein film. The thermal photomicrograph might then reveal the distribution and concentration of protein in the tissue section. It is entirely possible that a closer approach to the diffraction limit could be obtained by such an apparatus. At the present time, however, these possibilities have not been investigated.

Summary

Infrared microspectrophotometry is defined as infrared spectrophotometry of samples smaller than the slit of the monochromator. It therefore requires a microilluminator in the sample space. Present practice tends toward Schwarzschild-type totally reflecting microscope objectives for the microilluminator, with the sample in the dispersed radiation beam.

The minimum sample size for spectrophotometry from 2.5 to 15 μ is limited by diffraction to 6μ in diameter (about 10^{-9} gm.), although the practical limit due to instrumental limitations is about 30 μ in diameter (about 2×10^{-8} gm.) for extreme conditions. The difficulties in handling and containing such small liquid samples further increase the minimum sample size to about 1 μg., either as a solution or as a pure liquid. For fibers it is possible to use samples having a diameter equal to the diffraction limit.

The effects of the large angle of convergence on the radiation in the microilluminator are usually not important except for single-crystal spectra and for quantitative analysis.

The applications of infrared microspectrophotometry to biological problems are mostly concerned with identification of extracted compounds or studies of isolated chemical systems. Some interesting information can also be obtained from larger single cells and from whole-tissue preparations.

Recent developments in the field of infrared instrumentation suggest a bright future for infrared microspectrophotometry in biology.

References

1. Barnes, R. B., R. S. McDonald, V. Z. Williams & R. F. Kinnaird. 1945. Small prism infrared spectrometry. J. Appl. Phys. **16:** 77.
2. Anderson, D. H. & N. B. Woodall. 1953. Infrared identification of materials in the fractional milligram range. Anal. Chem. **25:** 1906.
3. Burch, C. R. 1947. Reflecting microscopes. Proc. Phys. Soc. London. **59:** 41.
4. Grey, D. S. & P. H. Lee. 1950. A new series of microscope objectives. J. Opt. Soc. Am. **39:** 719, 723.
5. Norris, K. P., W. E. Seeds & M. H. F. Wilkins. 1951. Reflecting microscopes with spherical mirrors. J. Opt. Soc. Am. **41:** 111.
6. Barer, R., A. R. H. Cole & H. W. Thompson. 1949. Infrared spectra with the reflecting microscope. Nature. **163:** 198.
7. Cole, A. R. H. & R. N. Jones. 1952. A reflecting microscope for infrared spectrometry. J. Opt. Soc. Am. **42:** 348.
8. Blout, E. R., G. R. Bird, & D. S. Grey. 1950. Infrared microspectroscopy. J. Opt. Soc. Am. **40:** 304.

9. Rupert, C. S. & J. Strong. 1950. The carbon arc as an infrared source. J. Opt. Soc. Am. **40:** 455.
10. Taylor, J. H., C. S. Rupert & J. Strong. 1951. An incandescent tungsten source for infrared spectroscopy. J. Opt. Soc. Am. **41:** 626.
11. Hall, M. B. & R. G. Nester. 1952. A zirconium concentrated-arc source for infrared spectroscopy. J. Opt. Soc. Am. **42:** 257.
12. Cashman, R. J. 1946. New photoconductive cells. J. Opt. Soc. Am. **36:** 356A.
13. Simpson, O. & G. B. B. M. Sutherland. 1951. The preparation and properties of photoconducting films of lead telluride. Trans. Roy. Soc. London. **243:** 547.
14. Coates, V. J., A. Offner & E. H. Siegler, Jr. 1953. Design and performance of an infrared microscope attachment. J. Opt. Soc. Am. **43:** 984.
15. Fraser, R. D. B. 1953. The interpretation of infrared dichroism in fibrous protein structures. J. Chem. Phys. **21:** 1511.
16. Caspersson, T. 1936. Über den chemischen Aufbau der Strukturen des Zellkernes. Skand. Arch. Physiol. **73:** Suppl. 8.
17. Wood, D. L. 1951. Infrared microspectrum of living muscle cells. Science. **114:** 36.
18. Wood, D. L. 1954. The identification of spleen propionylcholine by infrared microspectroscopy. Biochem. J. **58:** 30.
19. Gardiner, J. E. & V. P. Whittaker. 1954. The identification of propionylcholine as a constituent of ox spleen. Biochem. J. **58:** 24.
20. Blout, E. R., G. R. Bird & M. J. Abbate. 1952. Infrared microspectroscopy. III. A capillary cell for liquids. J. Opt. Soc. Am. **42:** 966.
21. Kaye, W. 1954. Near infrared spectroscopy. Spectrochim. Acta. **6:** 257.
22. Hecht, K. T. & D. L. Wood. 1956. The near infrared spectrum of the peptide group. Proc. Roy. Soc. London. **A235:** 174.
23. Bailly, R. 1950. Prismatic cleavage of molybdenite. Acta Cryst. **3:** 477.
24. Anon. July 30, 1956. Camera-like device sees a purple cow. Life. **41**(5): 6.
25. Wormser, E. Barnes Engineering Co. Stamford, Conn. Unpublished.
26. Pfund, A. H. 1939. Atmospheric contamination. Science. **90:** 326.

A BIBLIOGRAPHY OF INFRARED SPECTRA OF BIOCHEMICALS*

By Carl Clark and Maria Chianta
Naval Aviation Medical Acceleration Laboratory, Johnsville, Pa.

Introduction

The purpose of this work is to provide references to available infrared spectra of biochemicals. Although the bibliographic efforts, published and unpublished, of many people have been utilized, our work has not been reviewed by others prior to this publication. Hence, omissions can be expected. This is the first edition of a continuing task.

A reference is only part of the process of obtaining a spectrum. A larger goal of this work is to compile, or to contribute to the compilation of, the spectra themselves, in a form available to others and rapidly utilizable for the identification of unknown materials. Photocopying published material for the use of others requires securing permission from the copyright owners (Smith, 1955), which is obviously a large task. We need to obtain the cooperation of many people, particularly with regard to the contribution of unpublished spectra for compilation. It is hoped that the publication of this index and bibliography will stimulate investigators who have spectra of molecules for which inadequate or no references are given here to publish these spectra, or to send them to the National Research Council-National Bureau of Standards infrared catalogue (see below) or to send them to the authors.

In starting this work, an Index of Biochemicals was formulated, and then spectra for these biochemicals were sought. The index was to include all molecules and their common isolation derivatives that might be isolated by fractionation techniques from biological material and uniquely identified by infrared spectra. The latter criterion sets an upper limit on the molecular weight of 300 to 600, although this limit will be extended as spectra are recorded with higher resolution and greater quantitative accuracy. Abderhalden (1911–1915) in his nine-volume *Biochemisches Handlexikon* had as his purpose the compilation of the chemical, physical, and physiological properties of all molecules that occur in nature and of their direct derivatives of interest. In general, he excluded derivatives of derivatives. It is clear that our short index is not yet complete. We have excluded alkaloids, for example, and inorganic substances (Hunt, 1950; Miller, 1952). Many natural derivatives, or those useful in isolation procedures, are not yet included: for example, *N*-phosphorylated amino acids (Li, 1955); 2,4-dinitrophenylhydrazones (L. A. Jones, 1956); sodium salts of organic acids (Childers, 1955); and 3-phenyl-2-thiohydantoins of amino acids (Ramachandran, 1955). When all the spectra are available, it may be desirable to subdivide their indexing according to the known source of the biological sample: for example, mammalian tissue, mammalian urine, plant, or hydrolysis product. It is one goal of structural bio-

* The work on which this paper is based was supported in part by a grant from the American Cancer Society, Inc., New York, N. Y.

chemistry to describe the organism as a dynamic array of molecules and atoms in space. With present techniques, the extent of detail of this description can rarely reach one microgram of a particular compound per kilogram of tissue, or one microgram per liter of biological fluid. It is expected that many new biochemicals in lower concentrations, with molecular weights between those of the small metabolites and the biological macromolecules, will be described by improved techniques. Initial fractionation and infrared identification studies will, of course, emphasize those molecules present in higher concentrations.

Nomenclature

The nomenclature of this index consists largely of trivial names, although systematic names are used for the steroids and for certain of the less common biochemicals. A recent biochemistry text or a dictionary such as that of Heilbron (1953) may be used to identify these trivial names. It is hoped that the number of commonly used trivial names, representing structures that must be specifically memorized by each student, will be progressively reduced in teaching and in literature. Some aspects of systematic nomenclature have not been established by international agreements; the biochemist as well as the organic chemist would profit from such agreements. The nomenclature studies of committees of the American Chemical Society, illustrated by usage in *Chemical Abstracts*, are helpful. For "resonating" and tautomeric structures, it will be helpful to agree on a single "index name." For purposes of cross reference, a molecular serial-number designation may be desired; a wide use of the system of serial numbers that the Chemical-Biological Coordination Center of the National Academy of Sciences-National Research Council is establishing would be more convenient than a variety of serial-number codings. A number-letter symbolism of systematic nomenclature, adaptable to machine representation, has been developed for the Center and is described by Morgan (1947) and in *A Method of Coding Chemicals for Correlation and Classification*, National Research Council, Washington, D. C., 1950. Dyson (1947, 1949) has proposed another systematic symbolism, and there have been other attempts to increase the "information density" of chemical nomenclature. In order to develop improved nomenclature for complex molecules, we feel that it is important for students and others to begin to use these "machine language" methods. To speak or to write the name of a macromolecule in terms of its chemical groups and their spatial configurations, even with code designations, will be such a slow procedure that most of our utilization of such structural names will undoubtedly involve the much faster machine methods (Opler, 1956).

Molecular names should be sufficiently specific to enable other investigators to obtain the same spectrum of a sample of the same designation prepared in the same way. One must be particularly careful in the use of trivial designations for large molecules: there are many possible lecithins, trypsins, polyglutamic acids (Ambrose, 1950), and deoxyribonucleic acids. A molecule and its hydrate, steric isomer, or salt may have certain properties that are very

similar, but the infrared spectra are generally quite different. Hence extent of hydration, the configurations at all asymmetric carbon atoms and at double bonds, and the proportions of the components of a salt should be stated in the name. Crystalline polymorphism also produces spectral changes (Ebert, 1952; Kendall, 1953b), so such forms, which differ in intermolecular structure, should be specified. Harris (1953) and Michelson (1954) show that the cytidylic acid isomers *a* and *b* each have two polymorphic forms, crystallizing from water or from alcohol, for which the designations a_{H_2O} and $a_{alc.}$ and b_{H_2O} and $b_{alc.}$ were suggested. These polymorphs have distinct infrared spectra. Another example of the effect of polymorphism is described by R. N. Jones (1956) for the α and β polymorphs of stearic acid. It is expected that an intensified study of organic crystals will reveal many instances of polymorphism, because many of these molecules have weak intermolecular forces and hence are probably capable of packing in space in several configurations of about equal stability. A general designation for polymorphs is desirable. We recommend that polymorphic crystalline forms of molecules otherwise called by the same name be designated by giving in parentheses following the name the interplanar crystalline spacings determined from the three strongest X-ray diffraction powder pattern lines.

Sources of Data

General reviews of the applications of infrared spectroscopy to biology have been presented by Freeman (1956) and by Clark (1955). The review by R. N. Jones (1956) is excellent, particularly for chemical interpretations of spectra. The series of formerly annual, now semiannual, reviews on infrared spectroscopy by Robert Gore in *Analytical Chemistry* includes references to many molecules. Other reviews have appeared in *Annual Reviews of Physical Chemistry* and *Progress in Biophysics and Biophysical Chemistry*. The recent review of polypeptides by Bamford (1956), which includes infrared spectra, was not available to us. The infrared bibliography of C. R. Brown (1954) was utilized. Landolt-Börnstein (1951) contains approximately 1000 spectra compiled by H. Seidel, with sections on the molecules of biochemical interest, and many additional references compiled by Maier, Mecke, Kerkhof, Pajenkamps, and Seidel. It would be helpful if more journals would use an "infrared spectra" subject heading in the indices, with entries as to the molecules studied, as does the decennial index of *Applied Spectroscopy*, vol. 10, December 1956.

Our most useful source of references was the National Bureau of Standards compound file, which we reviewed at the Bureau. This file now contains about 17,500 molecular name cards giving references, largely from eighteen journals, to reports of infrared studies. The file is being compiled by Francis A. Smith, James F. Stewart, and E. Carroll Creitz, to whom we express our appreciation.

The several catalogues of spectra have been well illustrated by Brügel (1954). The API Research Project 44 *Catalog of Infrared Spectrograms**, compiled

* American Petroleum Institute, 50 West 50th St., New York, N. Y

under the direction of F. D. Rossini of the Carnegie Institute of Technology, was reviewed through spectrum 1661AA (Rossini, 1951). The Sadtler Standard Spectra* were reviewed through spectrum 7750CA. It is likely that, due to differences in nomenclature and other indexing problems, some of Sadtler's spectra of biochemicals are not referenced. The Sadtler catalogue is scheduled to grow at a rate of 1800 spectra per year. A "midget edition," with three spectra per $8^1{}_2$ in. $\times$ 11 in. page, is now available. Sadtler is considering the publication of a midget edition of the spectra of the biochemicals. These spectra include a range of 5000 to 650 cm.$^{-1}$ (2 to 15.4 μ). The American Society for Testing Materials† utilizes the Wyandotte-ASTM punched-card infrared spectrum index originated by Kuentzel (1951), which gives the positions of strong bands, but does not show the entire spectrum. The spectra abstracted from the literature by volunteers have been reviewed through abstracted spectrum 2678EA. Punched cards indexing the API, Sadtler, and NRC-NBS infrared spectra may also be obtained from ASTM, and ASTM has recently completed an empirical formula-name index of 13,899 IBM cards, which can be used to locate these and the abstracted literature spectra.

The National Bureau of Standards-National Research Council (NRC-NBS) punched cards‡ are of two types: compound cards, with spectra and references, reviewed through 584 DA, and bibliography cards, with abstracts (see *Chemical Engineering News*, Vol. 30, p. 3444, 1952). Creitz invites those with standard spectra, either not previously reported or better than those in the literature, to submit them for publication by the NRC-NBS method. The "Documentation of Molecular Spectroscopy" (DMS) system§ will publish 1600 spectral cards and 400 literature cards per year (Thompson, 1955; *Applied Spectroscopy*, Vol. 10, p. 104, 1956). Publication has commenced, but we have not yet seen an index of these spectra. The range of these various catalogue spectra is generally at least 4000 to 670 cm.$^{-1}$

Unfortunately, in the present paper it has not been possible to indicate the methods of preparation for the samples whose spectra are reported.

Inadequacies of Data

Problems of nomenclature discussed above have emphasized the fact that the name given to any molecule should be at least as specific as its spectrum. Sample preparation should be specified, since changes in method of preparation affect spectra. Different axial units, sizes, and length-to-width ratios used by different authors complicate direct comparisons of spectra. R. N. Jones (1956) has illustrated these methods of plotting. Fine grids are commonly removed on publication. We have experimented with photocopying to give spectra of uniform size, and with double printing to replace fine grids, with some success. When this work is repeated with greater emphasis on quantitative aspects, such quantities as resolution, slit widths (Kostowski, 1956), and sample con-

* Sadtler Research Laboratories, 1517 Vine St., Philadelphia, Pa.
† 1916 Race St., Philadelphia, Pa.
‡ E. Carroll Creitz, National Bureau of Standards, Washington 25, D. C.
§ Butterworths Scientific Publications, 88 Kingsway, London, W.C. 2, England, and Verlag Chemie GmbH., Weinheim/Bergstrasse, West Germany.

centrations and thickness will be noted. A description of the comparison-beam sample, including its chemistry, its thickness, and its "scattering," should be given.

An inadequacy of many published infrared studies is that only a part of the spectrum, or perhaps only a table of wave lengths of a few bands, may be shown in order to substantiate a specific discussion, for example, of the correlation of a particular band position with the presence in the absorbing molecules of a particular chemical group. Yet, as far as identification of structures is concerned, the band-assignment approach can do no more than give aspects of the molecular structure, generally with some uncertainties. The full spectrum can provide a unique identification, thus giving the complete structure to the extent this has been recorded in the literature by workers using all available techniques. The partial spectrum and band correlations can provide only suggestions for parts of the structure. It is recommended that all those who are concerned with band-group correlations publish or make available their entire spectra. Band-group correlations are useful, indeed, if they advance our concepts of molecular structure; that is, if they involve careful analyses of band shifts with slight alterations of structure and of band intensity changes in terms of modifications of dipole moments, electric distributions, and the like. For molecules whose valence structures are not known, these correlations may provide valuable suggestions as to the correct structures. However, we recommend spectrum-molecular structure correlations and inclusive catalogues in addition, if not in preference, to band-group correlations.

This paper will be of no direct aid to an investigator who wishes to identify a spectrum of a biochemical. At present the best method for doing this is to utilize the ASTM infrared-spectrum index cards. Efforts will be made to see that all spectra for which references are cited here are included in the ASTM index. We have also requested the ASTM to authorize the sale of a "biochemical" subdivision of the spectrum index and of the formula-name index. We have found the spectrum index of Clark (1950b), which consists of the wave lengths and relative absorptivities of the three strongest bands between 1100 and 670 cm.$^{-1}$ (9 and 15 μ), to be useful in identifying pure compounds, and we may include such a spectrum index in a later report. A later report will also contain an atomic-formula index and a name index that is not subdivided by biochemical categories, as is the name index included here.

"Data Pools" of the Properties of Biochemicals

The infrared absorption characteristics of a molecule are but one property of many that should be known to describe the behavior of the molecule and to contribute to our understanding of it. Our larger goal is to contribute to the compilation of all properties; in order to increase the effectiveness of utilization of so many data, we feel, as many others do, that machine methods for storing and retrieving the material in these data pools should be used. In addition to the infrared files, ASTM has ultraviolet and X-ray diffraction files, which may be purchased. The DMS keysort card gives certain other physical properties in addition to the infrared spectrum. The Chemical-Biological Coordination Center program, mentioned above in the section on *Nomenclature*,

involves a coding of biological properties (Wood, 1956). Other compilations—of chemical properties by Heilbron (1953), Beilstein (1918 1956), Elsevier's *Encyclopedia* (1940 48), and Kirk and Othmer's *Encyclopedia* (1950–1957); of physical properties of chemicals, by Dreisbach (1955), Winchell (1954), and Wyckoff (1953); and of biological properties of chemicals by Abderhalden (1911 1915), Spector (1956), and Hoppe-Seyler Thierfelder (1955), to give a few examples must be incorporated into the data pools and translated into machine language. Our need is to establish the form of the data pools and of the machine language so that all scientists can contribute to and readily obtain and utilize information from such international pools in preference to scattered publication elsewhere. It is becoming increasingly apparent that it is not limitations of information availability but limitations of the rate of information transfer that most retard the advance of science. We must rely more and more upon machines as adjuncts to our brains to ensure system, to ensure speed, and to ensure inclusiveness.

Automatic Inclusive Small-Molecule Biochemical Analysis and Molecular Biology

A major application of infrared spectroscopy to biology is for the identification of biochemicals. For recognition of a pure compound, the ASTM spectrum index may be adequate if the compound is included in the catalogue. Our first need, then, is to ensure that spectra of all compounds of interest are included, based on several methods of sample preparation where necessary.

Once the sample is identified, we should provide a number of spectra for its quantitative analysis, and these spectra are a second need of the catalogue. However, biological fractionation products are usually not pure, but contain a mixture of biochemicals. The spectrum of such a mixture generally contains the bands of the separate components, although there may be some band displacements and even some new bands, representing new vibrational modes, due to interactions. These bands may overlap, obscuring one another. The spectrum indices are used with difficulty for identifications of even simple mixtures (however, see Baker, 1953). More information than that provided by the spectral positions of a few band maxima must be utilized in the analysis.

The information furnished by a spectrum may be considered in terms of the accuracies of measurements of intensities and spectral positions (King, 1953). However, for a particular molecule the relative intensities, including certain finite band widths, and the spectral positions are predetermined by the structure and cannot take on all possible values. Hence, the information content is less than would be indicated by the possible accuracy of recording. From another point of view, one may consider that a very limited amount of information is required of the spectrum: the identification and quantitative determination of the components. The source of the sample may significantly delimit the possible components. In addition to infrared spectra, other properties may be utilized in making automatic comparisons to the datum pools. Yet another point of view is that each property may be considered as a code whose informational content is determined by the uniqueness of identification within a particular data pool. If the identification is unique, one might,

for example, "read in" to the data-pool memory device an infrared spectrum and "read out" the expected X-ray diffraction powder pattern for the same substance. It is recognized that these considerations of information require the processing of many more numbers than those of a few band positions, and hence require more involved machine methods.

To gain experience and establish methods, we have semiautomatically converted infrared spectra to digital representation on IBM cards utilizing Benson-Lehner "Oscar" data reduction equipment. In this preliminary work we have punched absorptivities in three decimal digits and wave lengths at each 0.1 μ. King (1954) has carried out completely automatic digital recording of all the information of the infrared spectrum. We have developed the following equations for use with chemical systems that obey the absorption laws. In these equations, A is the absorbance of the unknown, a is the absorptivity of a component, b is the thickness of the unknown, c is the concentration of a component, σ is the wave number position in the spectrum, and n is the number of components.

(1) When the analysis is complete, the difference-spectrum between the unknown sample and the components in computed concentrations will average 0:

$$\int_\sigma \left[A(\sigma) - b\sum_{i=1}^{n} c_i a_i(\sigma) \right] d\sigma \equiv \int_\sigma \Delta(\sigma) d\sigma = 0$$

(2) When the analysis is complete, the squared difference-spectrum area will be minimized:

$$\int_\sigma \left[A(\sigma) - b\sum_{i=1}^{n} c_i a_i(\sigma) \right]^2 d\sigma \equiv \int_\sigma \Delta^2(\sigma) d\sigma = \text{minimum}$$

(3) By partial differentiation, equation 2 may be shown to yield n equations of the form:

$$\frac{1}{b} \int_\sigma A(\sigma) a_j(\sigma) d\sigma = \sum_{i=1}^{n} c_i \int_\sigma a_i(\sigma) a_j(\sigma) d\sigma$$

With the unknown and component spectra in digital form, we have carried out automatic solutions of EQUATION 3 with the IBM Card Programmed Calculator. Once these are programmed, automatic computation utilizing virtually all the information of the spectrum is quite feasible. For large numbers of spectra, reference to detailed digital standard spectra on punched cards will be too slow; consequently, we are very much interested in the development of computers having a large "memory" with rapid access. We acknowledge with thanks helpful discussions of this topic with Paul Lighty, Mortimer Rogoff, and J. William Brogan, Jr., of the Federal Telecommunications Laboratories, Nutley, N. J. Rogoff has reported on their work in this publication.

We have not yet carried out an automatic analysis of a multicomponent unknown that might include any one or more of a large number of components whose spectra are in machine memory. Our method will be initially to sub-

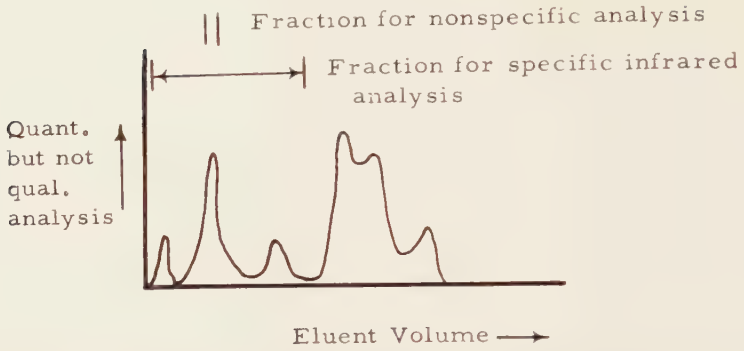


FIGURE 1. A comparison of the chromatographic eluent fraction volume suitable for the commonly used analytical methods, which cannot provide qualitative identification of the components and thus require their chromatographic resolution, and the fraction volume suitable for infrared analysis, which can provide both qualitative and quantitative information.

tract the spectrum of each compound in the catalogue at the 5 per cent concentration level and automatically to observe (1) whether the difference spectrum becomes negative at any point, ruling out that compound at that concentration, or (2) whether the slopes of the difference spectrum change sign at the bands of the compound or change magnitude by a "significant" amount. Once possible components are identified, their concentrations for optimum curve fitting would be solved, still automatically, by EQUATION 3. Each component would then be varied in concentration by a small amount, with the concentrations of the other components continually adjusted to maintain the criterion that the difference spectrum be a minimum. The magnitude of change of area of the squared difference spectrum would be used as a measure of the accuracy of the analysis of that component.

Mechanizations of fractionation procedures are equally feasible, and it is by a combination of these with automatic infrared spectra-memory-computer analysis methods that we are working to attain "automatic inclusive small-molecule biochemical analyses." FIGURE 1 illustrates the point that in the analysis of chromatographic eluents, the utilization of infrared spectra, which are both qualitatively and quantitatively specific, instead of the commonly used nonspecific quantitative methods such as the ninhydrin reaction or total near-ultraviolet absorption, makes it possible to analyze larger eluent volumes or to require less complete fractionations. If a fraction gives a spectrum which, because of broad bands, cannot be analyzed accurately, further fractionation would be carried out. For macromolecules, these fractionations will include stepwise hydrolyses. In distinction to most biochemical work, in which only a few fractions are studied, we shall attempt to study all fractions to attain inclusive biochemical description to the limits of accuracy of these methods; such an attempt is feasible only by utilizing machine methods.

It is clear that improvements in instrumentation and in the standard data catalogue are required for this program. Improvements in the microtechniques of fractionation, of sample preparation, and of obtaining the infrared spectra are desirable to enable us to study biological samples in the milligram to

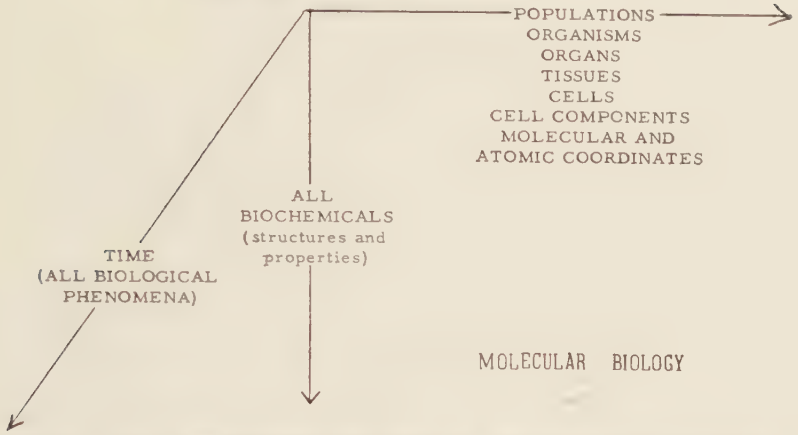


FIGURE 2. A suggested structure for cataloguing research in molecular biology. This structure is suitable for machine representation of the coordinates and for machine correlations.

microgram weight range. We recommend the development of infrared instruments with cathode-ray tube display (reviewed by Powell, 1952) for sample preparation and compensation beam adjustments, and with connection to a memory-computer device.

As an automatic analysis is carried out, it should be possible to display on the cathode-ray tube the memory spectra, in any combinations and concentrations, and the difference spectrum. It is not unreasonable to visualize that commercial infrared instruments might be supplied with such a memory-computer device, already containing the standard spectra. Digital recording eliminates the problems of spectral size and axis scales, for the digital data can be supplied, possibly through a computer link, to equipment which will automatically display the analogue curve on any reasonable size and coordinate scales. This and other advantages of digital recording of infrared spectra are presented by King (1954).

It is foreseeable, then, that the biologist will be freed from the specialized training and the long time now required for detailed small-molecule biochemical analyses of his material. The investigator could therefore add detailed biochemical descriptions to all of his other studies, making it possible to relate molecular changes to biological phenomena. In FIGURE 2 we have schematically represented such relationships, the study of which has been called "molecular biology." Our present work is directed toward the study of the detailed biochemical changes that accompany exposure to acceleration and to high-energy radiations, but we look forward to the day when inclusive biochemical analysis machines will be used by all hospitals and biologists. This may bring closer the day of detailed multivariable analysis of biological phenomena, a "biology by computer" that, because of the complexities involved, must come before we can achieve a quantitative understanding of life.

An Index of Biochemicals with References to Their Infrared Spectra

Abbreviations and Indexing Methods. The American Society for Testing Materials identifies various sources of physical data on its IBM-type index cards by a serial number and two letters. The final letter A, in column 80 of the IBM cards, signifies infrared data. The index cards may be obtained from ASTM; the spectra may be obtained from the literature or from the organizations listed above in the section on *Sources of Data*. The letters have the following meanings:

AA—American Petroleum Institute Research Project 44 Catalog of Infrared Spectrograms.

CA—Sadtler Standard Infrared Spectra.

DA—National Research Council-National Bureau of Standards Infrared Punch Cards.

EA—Literature spectra indexed by volunteer abstractors organized by the American Society for Testing Materials.

In this paper an additional letter code is used:

LB I—Landolt-Börnstein, Molekeln I (see the Bibliography).

LB II—Landolt-Börnstein, Molekeln II.

The page on which the spectrum appears is given after LB I or LB II.

Published literature is cited by first author and year, with such additional designation as a, b, and c, if there are several papers by this author during this year. The detailed reference is given in the Bibliography. In parentheses following the reference, the sample preparation and wave length range of the spectrum are generally given. Two references for the same spectrum are hyphenated.

The sequence of chemical names in each section is in accordance with the parent-compound method of *Chemical Abstracts*, with the alphabetical sequence of the parent compounds taking precedence over the alphabetical sequence of the substitution groups. Steric designations, such as α, L, or *cis*, do not affect the parent-compound sequence, but prefix designations of bond structure changes (for example epi, and nor) are alphabetized according to these designations. The multiple group designations (di, tri, poly) are placed in numerical sequence under the alphabetical position of the group. A later edition of this work will have a combined name index and an atomic formula index.

Amino Acids, Peptides, and Derivatives with up to Two Atoms Substituted

Acrylic acid, amino-.

Alanine,

D- or L-: LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ); Koegel, 1955 (KBr disk, 2-8 μ table); Lenormant, 1946 (5–8 μ); Lenormant, 1952a (D_2O solutions, varying *p*H, 5.5–7.5 μ).

DL-: 794 CA; 727 EA-Randall, 1949 (p. 119, mull, 2–9 μ); LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Lacher, 1954 (in fused $SbCl_3$, 1–12 μ); Margoshes, 1954 (C=O, 1.21 Å., 1580 cm. $^{-1}$); Thompson, 1950 (5.9–14.3 μ line diagram).

DL-, hydrochloride: 993 EA-Randall, 1949 (p. 226, mull, 2–9 μ).

DL-, sodium salt: Gore, 1949 (water, D_2O, DCl, and NaOD solutions, 2.5–15.4 μ).

Alanine, alanyl-, LL, DD, DL, LD: Ellenbogen, 1956c (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Alanine, tri-, 3L, LLD, LDL, DLL, 3D: Ellenbogen, 1956a (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Alanine, tetra-, 4L, LLDL, LDLL, DLLL: Ellenbogen, 1956a (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Alanine-poly-,

DL-: Elliott, 1954 (film cast from water, 6.7–12.5 μ).

L-: Astbury, 1948 (mull, 5.9–14.3 μ); Elliott, 1954 (α or β forms, cast from dichloroacetic acid or stretched in steam, 6.7–12.5 μ).

Alanine, alanyllysyl-, 3L, LLD, LDL, monohydrochlorides: Ellenbogen, 1956b. (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Alanine, alanyllysyl-di-, 4L, dihydrochloride: Ellenbogen, 1956b (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Alanine, L-alanyl-D-lysyl-di-L-, monohydrochloride: Ellenbogen, 1956b (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Alanine, alanyllysyl-tri-, 5L, LDLLL, monohydrochloride: Ellenbogen, 1956b (Fluorolube mull, 2–7 μ; Nujol mull, 7.4–16 μ).

Alanine, α-glutamyl-, LL, LD: Ellenbogen, 1956b (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

D- or L-Alanine, glycyl-: Ellenbogen, 1956a (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

β-Alanine: 795 CA; 744 EA-Randall, 1949 (p. 123, mull, 2–9 μ); 2195 EA-Toribara, 1954 (KBr disk, 2–16 μ).

Anserine.

Arginine,

D- or L-: 785 CA; 5448 CA; Larsson, 1950 (mull, 2–15 μ line diagram and table); Witkop, 1954a (mull, 5.9–6.6 μ table).

D- or L-hydrochloride: 5558 CA; Buswell, 1942 (film, 2.2–4.2 μ); Klotz, 1948 (film from evaporated water solution, 5–10 μ); Koegel, 1955 (KBr disk, 2–8 μ table).

Arginine phosphate.

Asparagine: Davies, 1953 (CCl_4 suspension and D_2O solution, 3.4–7.7 μ).

Aspartic acid,

D- or L-: 5560 CA; 7919 CA; Koegel, 1955 (KBr disk, 2–8 μ table); Lenormant, 1946 (5–8 μ).

DL-: 5425 CA; 734 EA-Randall, 1949 (p. 121, mull, 2–9 μ); LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Thompson, 1950 (5.9–14.3 μ line diagram).

L-Aspartic acid, poly-: 61 EA-Berger, 1951 (film from dimethylformamide solution, 5.6–9.5 μ).

Aspartic acid, carbamyl-.

Azaserine: *O*-diazoacetyl-L-serine: Fusari, 1954 (mull, 2–15 μ); Moore, 1954 (mull, 2–15 μ).

Butanoic acid, 2-amino-, L-: 2954 CA.
Carnosine.
Citrulline, 2-amino-5-ureidopentanoic acid: Koegel, 1955 (KBr disk, 2–8 μ table).
DL-: 2122 CA.
Cystathionine.
Cysteic acid.
Cysteine, L-hydrochloride: 326 CA; 1000 EA-Randall, 1949 (p. 227, mull, 2–9 μ); Koegel, 1955 (KBr disk, 2–8 μ table).
Cystine,
D-, or L-: 323 CA; 735 EA-Randall, 1949 (p. 121, mull, 2–9 μ); Cymerman, 1951 (C—S band, 674 cm.$^{-1}$; S—S band, 454 cm.$^{-1}$); Koegel, 1955 (KBr disk, 2–8 μ table); Lenormant, 1946 (5–8 μ); Otey, 1954 (Nujol and hexachlorobutadiene mulls, 2–15 μ).
D- or L-, hydrochloride: 999 EA-Randall, 1949 (p. 227, 2–9 μ).
L, D, DL, meso-cystine: Wright, 1937 (6–23 μ).
L-Cystine, D-cystinyl- and L-cystinyl-D-cystine: Otey, 1954 (Nujol and hexachlorobutadiene mulls, 2–15 μ).
L-Cystine, L-cystinyl-: Otey, 1954 (Nujol and hexachlorobutadiene mulls, 2–15.5 μ).
Ergothioneine.
Glutamic acid,
D- or L-: 2124 CA; 5561 CA; 746 EA-Randall, 1949 (p. 123, mull, 2–9 μ); LB I 444 (line diagram-Wright, 1939 (powder, table, 3–24 μ); Freeman, 1956 (mull, 5–15.5 μ); Koegel, 1955 (KBr disk, 2–8 μ table).
DL-: LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Thompson, 1950 (5.9–14.3 μ line diagram).
D- or L-hydrochloride: 5429 CA; 998 EA-Randall, 1949 (p. 227, mull, 2–9.2 μ).
Glutamic acid, 2-alanyl-, LL, LD: Ellenbogen, 1956b (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).
Glutamic acid, glutamyl-, LL, DD: Ellenbogen, 1956b (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).
Glutamic acid, poly-: Ambrose, 1950 (natural and synthetic samples, 5.5–10 μ, polarization spectra); Blout, 1956 (oriented film from dioxane-water solution, 3 μ, 6 μ, bands, polarization study).
Glutamic acid, carbamyl-.
Glutamine, D- or L-: 2125 CA; 5454 CA; Davies, 1953 (CCl_4 suspension and D_2O solution, 3.4–7.7 μ).
Glutathione, 7391 CA; Cymerman, 1951 (C—S band, 691 cm.$^{-1}$); Freeman, 1956 (film, 5–15.5 μ).
Glycine: 6475 CA; 512, 512a-DA; 2186 EA-Haeskaylo, 1954 (film from water slurry, 2–15.3 μ); LB I 443-Klotz, 1948 (film from water solution, 5–10 μ); LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Blout, 1952a (mull, 2.8–16 μ); Gore, 1949 (mull and H_2O, D_2O, HCl, DCl, NaOH, and NaOD solutions, 2.5–15.4 μ); Koegel, 1955 (KBr disk, 2–8 μ table); Lenormant, 1946 (5–8 μ); Lenormant, 1952b (film

from water slurry, 2–15.3 μ); Thompson, 1950 (5.9–14.3 μ line diagram).

hydrochloride: LB I 443-Klotz, 1948 (film from water solution, 5–10 μ).

Glycine, alanyl-.

D- and L-: Ellenbogen, 1956a (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

DL-: Lenormant, 1945 (5.7–7.9 μ).

Glycine, glycyl-: LB I 442-Kellner, 1941 (quartz prism, crystalline deposit, 2.9–3.6 μ); Abbott, 1953 (crystalline film, 2.9–3.2, 5.7–7.1 μ); Blout, 1952a (mull, 2.8–16 μ); Lenormant, 1945 (5.7–7.9 μ); Lenormant, 1952b (5.5–8 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.6, 5.6–8.3 μ line diagram).

Glycine, glycylglycyl-: 441 EA-Gäumann, 1952 (mull, 2–16 μ); Abbott, 1953 (crystalline film, 2.9–3.2, 5.7–7.1 μ); Blout, 1952a (mull, 2.8–16 μ); Lenormant, 1945 (5.7–7.9 μ); Lenormant, 1952b (5.5–8 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.6, 5.6–8.3 μ line diagram).

Glycine, triglycyl-: Abbott, 1953 (crystalline film, 2.9–3.2 μ, 5.7–7.1 μ); Blout, 1952a (mull, 2.8–16 μ); Lenormant, 1952b (5.5–8 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.6 μ, 5.6–8.3 μ line diagram).

Glycine, penta-: Blout, 1952a (mulls, 2.8–16 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.6 μ, 5.6–8.3 μ line diagram).

Glycine, hexa-: Blout, 1952a (mull, 2.8–16 μ).

Glycine, poly-: Astbury, 1948 (mull, 5.9–14.3 μ); Becker, 1954 (mull, 5.5–7.5 μ); Blout, 1952a (mull and film from dichloroacetic acid solution, 2.8–16 μ); Elliott, 1954 (film from trifluoroacetic acid solution, 6.7–12.5 μ); Hurd, 1953 (film from formic acid solution, 2–14 μ line diagram); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.6 μ, 5.6–8.3 μ).

Glycine, glycyl-D-alanyl-: Ellenbogen, 1956a (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Glycine, glycyl-L-alanyl-: Ellenbogen, 1956a (Fluorolube mull, 2–7.4 μ; Nujol mull, 7.4–16 μ).

Glycine, glycyl-D-leucyl-: Blout, 1952a (mull, 2.8–16 μ).

Glycine, glycylglycyl-DL-leucyl-: Blout, 1952a (mull, 2.8–16 μ).

Glycine, leucyl-,

L-: Blout, 1952a (mull, 2.8–16 μ).

DL-: Lenormant, 1945 (5.7–7.9 μ).

Glycine, leucylglycyl-,

D- or L-: Blout, 1952a (mull, 2.8–16 μ); Lenormant, 1945 (5.7–7.9 μ).

DL-: Lenormant, 1945 (5.7–7.9 μ).

Glycine, L-leucyl-L-leucyl-: Blout, 1952a (mull, 2.8–16 μ).

Gramicidin: Klotz, 1949 (film from ethanol solution, 2–10 μ).

Hippuric acid: 2802 CA; Bellamy, 1954 (p. 242, 5.5–15 μ).

sodium salt, hydrate: 720 EA-Randall, 1949 (p. 116, 2–9 μ).

Histidine: 5456 CA; Koegel, 1955 (KBr disk, 2–8 μ table).

D- or L-, hydrochloride: 5570 CA; Larsson, 1950 (mull, 2–15 μ line diagram and table).

D- or L-, dihydrochloride: 5457 CA.

Histidine, 1-methyl-.

Histidine, 3-methyl-: Tallan, 1954 (mull, 2–14.5 μ).

Homocysteine.

Homocysteine, *S*-adenosyl-.

Homocystine: 2294 CA; Koegel, 1955 (KBr disk, 2–8 μ table); Otey, 1954 (Nujol and hexachlorobutadiene mulls, 2–15.5 μ).

Homoserine: Koegel, 1955 (KBr disk, 2–8 μ table).

Isoleucine,

D- or L-: 787 CA; 736 EA-Randall, 1949 (p. 121, mull, 2–9 μ); Koegel, 1955 (KBr disk, 1–16 μ).

DL-: 5458 CA; Lacher, 1954 (in fused $SbCl_3$, 1–12 μ).

L- and DL-, acetylation products: Darmon, 1948 (mull, 5.6–15.4 μ).

Kynurenine.

Lanthionine.

Leucine,

D- or L-: 790 CA; 5562 CA; 5592 CA; LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Koegel, 1955 (KBr disk, 2–8 μ table); Lacher, 1954 (in fused $SbCl_3$, 1–12 μ); Larsson, 1950 (mull, 2–15 μ); Thompson, 1950 (5.9–14.3 μ line diagram).

DL-: 789 CA; LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table).

L- and DL-, acetylation products: Darmon, 1948 (mull, 5.6–15.4 μ).

L-Leucine, glycine-, 1,1-copolypeptide: Becker, 1954 (mull, 5.5–7.5 μ).

L-Leucine, glycyl-: Blout, 1952a (mull, 2.8–16 μ).

D-Leucine, glycylglycyl-: Blout, 1952a (mull, 2.8–16 μ).

L-Leucine, glycyl-L-leucyl-: Blout, 1952a (mull, 2.8–16 μ).

L-Leucine, glycyl-L-leucyl-glycyl-: Blout, 1952a (mull, 2.8–16 μ).

L-Leucine, L-leucyl-: Blout, 1952a (mull, 2.8–16 μ).

L-Leucine, poly-: Becker, 1954 (mull, 5.5–7.5 μ); Blout, 1952a (mull and film from benzene solution, 2.8–16 μ).

L-Leucine, L-leucylglycyl-: Blout, 1952a (mull, 2.8–16 μ).

L-Leucine, L-ornithyl-: Thompson, 1950 (2.8–3.3 μ, 5.7–14.3 μ line diagram).

Lysine,

D- or L-: 788 CA; 793 CA.

D- or L-, hydrochloride: 5563 CA; Koegel, 1955 (KBr disk, 2–8 μ table).

DL-, hydrochloride: 435 CA.

Lysine, poly-: Klotz, 1949 (film from water solution, 2–10 μ).

Lysine, 4-hydroxy-.

Mercapturic acid; acetylcysteine.

Methionine,

D- or L-: 5564 CA; 5565 CA; Cymerman, 1951 (C—S band, 688 $cm.^{-1}$); Koegel, 1955 (KBr disk, 2–8 μ table).

DL-: 131 CA; 139 EA-Short, 1951 (mull, 5.5–15 μ); Bellamy, 1954 (p. 240, 3.1–15 μ); Thompson, 1950 (5.9–14.3 μ line diagram).

Methionine, *S*-adenosyl-.

Norleucine,
D- or L-: 791 CA; LB I 443-Klotz, 1948 (thin film, 5–10 μ); Koegel, 1955 (KBr disk, 2–8 μ table).
DL-: 437 CA; 5461 CA; Thompson, 1950 (5.9–14.3 μ line diagram).
Norleucine, poly-: Hurd, 1953 (film from formic acid solution, line diagram, 2–14 μ).
Octopine.
Ornithine,
D- or L-: Koegel, 1955 (KBr disk, 2–8 μ table).
D- or L-, hydrochloride: 2123 CA.
D- or L-, dihydrochloride: Larsson, 1950 (mull, 2–15 μ line diagram and table).
L-Ornithine, 5-(L-valyl -: Thompson, 1950 (2.8–3.3, 5.7–14.3 μ line diagram).
Phenylalanine,
D- or L-: 792 CA; 5556 CA; 5557 CA; LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Koegel, 1955 (KBr disk, 2–8 μ table).
DL-: 436 CA; 745 EA-Randall, 1949 (p. 123, mull, 2–9 μ); LB I 444 (line diagram -Wright, 1939 (powder, 3–24 μ table); Blout, 1950a (mull).
Phenylalanine, poly-: Hurd, 1953 (films from *m*-cresol solution, benzene solution, and formic acid solution, 2–14 μ line diagrams).
Phenylalanine, 3,4-dihydroxy-.
Proline,
D- or L-: 1005 CA; 741 EA-Randall, 1949 (p. 228, mull, 2–9 μ); Buswell, 1942 (film, 2.7–4.1 μ); Koegel, 1955 (KBr disk, 2–8 μ table); Lenormant, 1946 (5–8 μ).
D- or L-, hydrochloride: 1005 EA-Randall, 1949 (p. 228, mull, 2–9 μ).
Proline, 4-hydroxy-: 786 CA; Koegel, 1955 (KBr disk, 1–16 μ).
L-Proline, L-leucyl-, anhydride: J. L. Johnson, 1951 (mull, 2.9–3.3, 6.2–14.3 μ).
Sarcosine: 742 EA-Randall, 1949 (p. 122, mull, 2–9 μ).
hydrochloride: 1001 EA-Randall, 1949 (p. 288, mull, 2–9 μ).
Serine,
D- or L-: 5566 CA; 5567 CA; Koegel, 1955 (KBr disk, 2–8 μ table).
DL: 454 CA; LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table).
L-Serine, *O*-glycyl-,
monohydrochloride: Moore, 1954 (mull, 2–15 μ).
DL-serine, *O*-glycyl-, hydrochloride: Moore, 1954 (mull, 2–15 μ).
Serine, phospho-.
Threonine,
D- or L-: 784 CA; Gore, 1949 (mull and H_2O, D_2O, DCl, and NaOD solutions, 2.5–15.4 μ); Koegel, 1955 (KBr disk, 1–16 μ); Lacher, 1954 (in fused $SbCl_3$, 1–12 μ); Margoshes, 1954 (C=O, 1.25 Å., 1620 cm.$^{-1}$).
Thyroxine, DL-: 2482 CA; 335 EA-Wang, 1952 (mull, 5–15 μ).
Tryptophan: Lenormant, 1946 (5–8 μ).
D- or L-: 5569 CA; Koegel, 1955 (KBr disk, 2–8 μ table).
DL-: 432 CA.
Tryptophan, poly-: Hurd, 1953 (film from ethanol solution, 2.8–14 μ).
Tyrocidine: Klotz, 1949 (films from ethanol solution, 2–10 μ).

Tyrosine: Lenormant, 1946 (5–8 μ).
D- or L-: 764 CA; 5568 CA; 743 EA-Randall, 1949 (p. 123, mull, 2–9.2 μ); LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Koegel, 1955 (KBr disk, 2–8 μ table); Thompson, 1950 (5.9–14.3 μ line diagram).
D- or L-, hydrochloride: 1007 EA-Randall, 1949 (p. 229, mull, 2–9.2 μ).
hydrochloride: Fraser, 1950 (microcrystal, 2.8–3.8 μ, 5.9–14.3 μ polarized).
L-Tyrosine, glycyl-: Lenormant, 1945 (5.7–7.9 μ).
Tyrosine, 3,5-diiodo-: 2481 CA.
Valine,
D- or L-: LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Koegel, 1955 (KBr disk, 2–8 μ table); Larsson, 1950 (sublimate film, 2–8 μ; mull, 2–15 μ); Wright, 1955 (2 per cent in aqueous solution, 2.5–11 μ).
DL-: 453 CA; 728 EA-Randall, 1949 (p. 119, mull, 2–9.2 μ); LB I 444 (line diagram)-Wright, 1939 (powder, 3–24 μ table); Lacher, 1954 (in fused $SbCl_3$, 1–12 μ); Thompson, 1950 (5.9–14.3 μ line diagram).
D- or L-, hydrochloride: LB I 443-Klotz (film from water solution, 5–10 μ); Klotz, 1948 (5–10 μ); Larsson, 1950 (mull, 2–15 μ line diagram and table).
Valine, DL-leucyl-: Larsson, 1950 (mull, 2–15 μ).
Valine, L-prolyl-: Thompson, 1950 (2.8–3.3, 5.7–14.3 μ line diagram).
Valine, valyl-,benzoyl methyl esters: Hinman, 1950 (mulls, 2.8–16 μ).

Carbohydrates and Derivatives with up to Three Atoms Substituted

Acetone, dihydroxy-.
Allose.
Altrose.
Arabinose,
D- or L-: 1043 CA; LB II 646-Kuhn, 1950 (mull, 7.8–15 μ); Barr, 1940 (film from water solution, 1.8–2.6 μ); Rogers, 1938 (water solution, 2–11 μ table); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 6.8–18.8 μ).
α- and β-D-Arabopyranose: Barker, 1954b (mull, 10.5–13.3 μ table).
Arabinose-5-phosphate.
Cellobiose: 1020 CA; LB II 647-Kuhn, 1950 (mull, 7.8–15 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 6.8–18.8 μ).
Chondroitin sulfuric acid: Orr, 1954 (two forms, films cast from aqueous solution, 5.7–15 μ).
Chondrosamine; 2-amino-D-galactose.
Chondrosine.
Erythritol: 1030 CA; LB II 647-Kuhn, 1950 (film from methanol solution, 7.8–15 μ).
Erythrose.
Fructose, D- or L-: 2755 CA; Barr, 1940 (film from water solution, 1.8–2.6 μ); Rogers, 1938 (water solution, 2.8–11 μ table).
Fructose-1,6-diphosphate.

Fructose-6-phosphate.
Fucose, D- or L-: Kuhn, 1950 (mull, 7.8–15 μ).
Galactose: Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8 3.3, 6.8–18.8 μ).
D- or L-: 989 CA; LB II 646-Kuhn, 1950 (mull, 7.8 15 μ); Rogers, 1938 (water solution, 2–11 μ table).
α- and β-D-Galactopyranose: Barker, 1954b (mull, 10.5 13.3 μ table).
Galactosamine.
Galactosamine, *N*-acetyl-.
Galacturonic acid: Ultee, 1955 (mull, 5.5–6.5 μ).
Gentianose.
Gentiobiose.
Gluconic acid, D- or L-: 8335 CA.
Gluconic acid, 6-phospho-.
Glucosamine, hydrochloride: 5122 CA; 520 DA; Barker, 1954a (table); Kuhn, 1950 (mull, 8–15 μ).
Glucosamine, *N*-acetyl-, D- or L-: 5119 CA.
Glucose: 990 CA; Rogers, 1938 (water solution, 2–11 μ table); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 6.8–18.8 μ).
D-: 1040 CA; LB II 646-Kuhn, 1950 (mull, 2–15 μ); Barr, 1940 (film from water solution, 1.8 2.6 μ); Woernley, 1952 (film from water solution, 1–15 μ).
β-Glucose: Barr, 1940 (film from water solution, 1.8–2.6 μ).
α- and β-D-Glucopyranose: Barker, 1954a,b (10.5–13.3 μ table).
Glucose-1-phosphate.
Glucose-6-phosphate.
Glucose diphosphate; hexose diphosphate: Woernley, 1952 (film from water solution, 1–15 μ).
Glucurone, D-: 3274 CA.
Glucuronic acid,
D-: 1037 CA; LB II 646-Kuhn, 1950 (film from water solution).
calcium salt: 7230 CA.
sodium salt: 7231 CA.
potassium salt: 7232 CA.
Glyceraldehyde, DL-: 5180 CA.
Glyceraldehyde, 3-phospho-.
Glyceraldehyde, 1,3-diphospho-.
Glyceric acid, calcium salt: 6611 CA.
Glyceric acid, 2-phospho-: Woernley, 1952 ("phosphoglyceric acid," film from water solution, 1–15 μ).
Glyceric acid, 3-phospho-.
Glyceric acid, 1,3-diphospho-.
Glyceric acid, 2,3-diphospho-.
Glycerol: 169 CA; 326 DA; Shay, 1954 (liquid, 6.7–14.3 μ).
Glycerol, 1-phospho-.
Glycerose.
Glycolaldehyde.

L-Gulonic acid, 2-keto-.
Gulose.
Hyalobiuronic acid; aldobiuronic acid.
Hyaluronic acid: Orr, 1952 (film cast from aqueous solution, 5.6–12.5 μ); Orr, 1954 (film cast from aqueous solution, 5.7–15 μ).
Idose.
Inositols: Barker, 1954c (10.5–13.3 μ table); Shay, 1954 (mull, 6.7–14.3 μ).
i: 1060 CA; LB II 647-Kuhn, 1950 (7.8–15 μ).
d: 2778 CA.
Lactose: Manning, 1956 (KBr disk, 2–15.4 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 6.8–18.8 μ).
D-: 1049 CA; LB II 647-Kuhn, 1950 (mull, 7.8–15 μ).
β-Lactose: Barr, 1940 (film from water solution, 1.8–2.6 μ).
Lyxose, D-: Rogers, 1938 (water solution, 2–11 μ table).
Maltose: 1019 CA; LB II 647-Kuhn, 1950 (mull, 7.8–15 μ); Barker, 1954a (10.5–13.3 μ table); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 6.8–18.8 μ).
Mannitol: LB II 647-Kuhn, 1950 (film from methanol solution, 7.8–15 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 5.6–6.2, 7.7–20 μ).
D-: 992 CA.
Mannobiose.
Mannoheptulose.
Mannose: 993 CA; Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 6.8–18.8 μ).
D-: LB II 646-Kuhn, 1950; (mull and film from water solution, 2–15 μ); Rogers, 1938 (water solution, 2–11 μ table).
α- and β-D-Mannopyranose: Barker, 1954b (mull, 10.5–13.3 μ table).
Melibiose: 1050 CA; LB II 647-Kuhn, 1950 (mull, 7.8–15 μ).
α-Melibiose monohydrate and β-melibiose dihydrate: Fletcher, 1952 (mulls, 7.5–14 μ).
Mucic acid: 1682 EA-Tipson, 1953 (mull, 2–16 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 5.6–6.2, 7.7–20 μ).
ammonium salt: 7516 CA.
Primeverose.
Raffinose: 1032 CA.
L-: LB II 647-Kuhn, 1950 (mull, 7.8–19 μ).
Rhamnose: 1018 CA; Barr, 1940 (film from water solution, 1.8–2.6 μ).
L-: LB II 646-Kuhn, 1950 (mull, 7.8–15 μ).
Ribitol.
Ribose, D-: 1046 CA; LB II 646-Kuhn, 1950 (mull, 7.8–15 μ); Blout, 1949 (powder, 5–15 μ); Clark, 1952a (mull, 2–16 μ); Woernley, 1952 (film, 1–15 μ).
Ribose, 2-deoxy-.
Ribose-1-phosphate.
Ribose-5-phosphate.

Ribose-5-phosphate, 2-deoxy-.
Ribulose.
Ribulose-5-phosphate.
Ribulose diphosphate.
Rutinose.
Saccharic acid.
Sedoheptulose.
Sedoheptulose-7-phosphate.
Sorbitol: 991 CA; LB II 647-Kuhn, 1950 (mull, 7.8–15 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.3, 5.6–6.2, 7.7–20 μ).
Sorbose: 1069 CA.
L-: LB II 646-Kuhn, 1950 (mull, 7.8–15 μ).
Sucrose: Woernley, 1952 (film from water solution and dry powder from alcohol suspension, 1–15 μ).
Talose.
Threose.
Trehalose: 1058 CA; LB II 647-Kuhn, 1950 (mull, 7.8–15 μ).
Xylose: 1068 CA.
D-: LB II 646-Kuhn, 1950 (mull, 7.8–15 μ); Barr, 1940 (film from water solution, 1.8–2.6 μ).
L-: Rogers, 1938 (water solution, 2–11 μ table).
Xylulose.

Lipides and Derivatives with up to Three Atoms Substituted, Excluding Steroids

Acetic acid: 1220 AA; 76 CA; 52, 52c DA; 680 EA-Randall, 1949 (p. 103, liquid, 2–13 μ); LB I 429-Herman, 1938 (vapor, 138° C., 1–15.5 μ); Ard, 1951 (CCl_4 solutions, 2–7.2 μ, and CS_2 solutions, 7.2–15 μ, with and without a slight excess of triethylamine); Hadzi, 1953 (liquid, 6.6–20 μ; equimolar water solution, 6.6–14.3 μ); Pierson, 1956 (gas, 2–15 μ); Sternglanz, 1956 (glacial and 10 per cent water solution, 2–15 μ); Wall, 1939 (CCl_4 solution, 2.8–5 μ).
methyl ester: 2228 CA; Barnes, 1944 (p. 71, liquid, 5.5–9.8 μ); Torkington, 1945 (liquid, 3–20 μ).
sodium salt: 2579 EA-L. H. Jones, 1954 (residue from evaporated aqueous solution, room temp. and 80° K., 3.2–29 μ); 2624 EA- Childers, 1955 (mull, 7–15 μ).
Acetoacetic acid.
Arachidic acid: 5732 CA; 58 DA.
Arachidonic acid: Sinclair, 1952b (liquid, and solid at −196° C., 3.1–14.3 μ).
Behenic acid.
Butyric acid: 1227 AA; 125 CA; 54 DA; LB I 430-Herman, 1940 (vapor at 149° C., 1–13 μ).
sodium salt: 2626 EA-Childers, 1955 (mull, 7–15 μ).
Capric acid: 2705 CA; 3 DA; 41 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); Harple, 1952 (liquid, 2–14 μ).

Caproic acid: 308 CA; 1 DA; 39 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); Harple, 1952 (liquid, 2–14 μ); Jones, 1956 (p. 253, CS_2 solution, 7.4–15 μ); Klotz, 1948 (CCl_4 solution, 5–10 μ).

sodium salt: 2631 EA-Childers, 1955 (mull, 7–15 μ).

Caprylic acid: 2707 CA; 2 DA; 298 DA; 40 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); 692 EA-Randall, 1949 (p. 106, liquid, 2–15 μ); Harple, 1952 (liquid, 2–14 μ); Howton, 1951 (CCl_4 solution, 2–16 μ); Rigaux, 1954 (liquid 20° C., solid −20° C., 5.6–14.3 μ).

Castor oil: Kendall, 1953a (liquid, 2–15.5 μ).

L-α-Cephalin, dimyristoyl-: Baer, 1952 (mull, 2.5–15 μ); Marinetti, 1954 (mull 2.5–14.5 μ).

L-α-Cephalin, dipalmitoyl-: Baer, 1952 (mull, 2.5–15 μ).

L-α-Cephalin, distearoyl-: Baer, 1952 (mull, 2.5–15 μ).

Cerebron; *see* Phrenosin.

Cerebronic acid.

Cerotic acid.

Cetyl alcohol: 222 CA; Davies, 1940 (solid film, 2.7–3.5 μ); Neuilly, 1954 (liquid and solid, 5.8–14.3 μ); Swern, 1955 (CCl_4 solution, 2.6–3.2 μ).

Chaulmoogric acid.

Chimyl alcohol.

Choline.

Choline, acetyl-.

Choline, L-α-glycerophosphoryl-, cadmium chloride salt: Marinetti, 1954 (mull, 2.5–14.5 μ).

Crotonic acid: 749 CA.

1,3-Diolein: O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

D-1,2-Dipalmitin: Hanahan, 1954b ($CHCl_3$ solution, 2–15 μ).

1,3-Dipalmitin: O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

D-1,2-Dipalmitolein: Hanahan, 1954b ($CHCl_3$ solution, 2–15 μ).

1,3-Distearin: 89 CA; O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

Elaidic acid; *trans*-9-octadecenoic acid: 5329 CA; 46 DA; 1347 EA-Freeman, 1953a-Freeman, 1956 (CS_2 solution, 5–16 μ); 1762 EA-Ahlers, 1953 (CCl_4 solution, 2.5–7.5 μ; CS_2 solution, 7.5–15 μ); LB I 430-Rao, 1948 (CCl_4 solution, 2–16 μ); Benedict, 1950 (CCl_4 solution, 2–16 μ); Jones, 1952b (film, 7.1–8.5 μ); Shreve, 1950 (CS_2 solution, 2–15 μ); Sinclair, 1952b (CS_2 solution and solid film at 25° C., 3.1–14.3 μ).

Eleostearic acid, α-; 9 *cis*, 11 *trans*, 13 *trans*-octadecatrienoic acid: 1751 EA-Ahlers, 1953 (CCl_4 solution, 2.5–7.5 μ; CS_2 solution, 7.5–15 μ); 1866 EA-Bickford, 1953 ($CHCl_3$ solution, 1–12 μ).

β-; 9 *trans*, 11 *trans*, 13 *trans*-octadecatrienoic acid: 1752 EA-Ahlers, 1953 (CCl_4 solution, 2.5–7.5 μ; CS_2 solution, 7.5–15 μ); 1867 EA-Bickford, 1953 ($CHCl_3$ solution, 1–12 μ).

Ethanolamine: 123 CA; 511 DA; 930 EA-Randall, 1949 (p. 193, $CHCl_3$ solution, 2–12 μ); Bergmann, 1953 (CCl_4 solution, 3 μ table).

Ethanolamine, L-α-glycerylphosphoryl-.

Ethanolamine phosphoric acid.

Glycerol.

Glycerides, mono-: Kuhrt, 1952 (unsaturated: as liquids; saturated: as recrystallized melted films and as mulls, 2–16 μ).

Hentriacontane.

Heptacosane.

Hydnocarpic acid.

Isocaproic acid: 2706 CA.

Isovaleric acid: 1835 CA.

α-Kamlolenic acid: 18-hydroxy-9 *cis*, 11 *trans*, 13 *trans*-octadecatrienoic acid: Gupta, 1955 (CCl_4 solution, 2.8–7 μ; CS_2 solution, 7–15 μ).

Kerasin.

Lactobacillic acid: Hofmann, 1952 (film, 5–12 μ); Hofmann, 1953 (2–12 μ); Hofmann, 1954 (2–16 μ).

Lauric acid: 14 CA; 4 DA; 48 DA; 42 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); Davies, 1940 (solid film, 2.7–3.5 μ); Hadzi, 1953 (crystalline film, 6.6–20 μ); Harple, 1952 (2–14 μ); Jones, 1952b (7.1–8.5 μ).

L-α-Lecithin, dimyristoyl-: Baer, 1953 ($CHCl_3$ solution, 2.8–12 μ); Marinetti, 1954 (mull, 2.5–14.5 μ).

L-α-Lecithin, dioleyl: Baer, 1956 (2–15.5 μ).

L-α-Lecithin, dipalmitoleyl-: Hanahan, 1952 ($CHCl_3$ solution, 2–15 μ).

L-α-Lecithin, dipalmitoyl: Baer, 1952 ($CHCl_3$ solution, 2.8–12 μ); Hanahan, 1952 ($CHCl_3$ solution, 2–15 μ).

L-α-Lecithin, distearoyl-: Baer, 1953 ($CHCl_3$ solution, 2.8-12 μ).

Lecithins, lyso-;

α-Palmitoleyl-L-α-lecithin; monopalmitoleyllecithin: Hanahan, 1954a ($CHCl_3$ solution, 2–12 μ).

β-Palmitoyl-L-α-lecithin; monopalmitoyllecithin: Hanahan, 1954a ($CHCl_3$ solution, 2–12 μ).

Licanic acid.

Lignoceric acid.

Linoleic acid: 914 CA; 1758, 1761 EA-Ahlers, 1953 (liquid, 2.5–15 μ); Jones, 1952b (low temperature film, 7.1–8.5 μ); Jones, 1956 (p. 316, liquid film at 30° C. and crystalline film at −195° C., 6.7–15 μ); Sinclair, 1952b (liquid, and solid at −196° C., 3.1–14.3 μ); Vañadi, 1938 (1–9 μ); Walborsky, 1951 (CCl_4 solution, 2–10 μ; CS_2 solution, 10–16 μ).

Linolenic acid: 916 CA; 1756 EA-Ahlers, 1953 (liquid, 2.5–15 μ); Jones, 1952b (low temperature film, 7.1–8.5 μ); Sinclair, 1952b (liquid, and solid at −196° C., 3.1–14.3 μ).

Linseed oil: Shreve, 1952 (liquid, 2.5–15 μ).

1-Monomyristin: O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

1-Monoolein: O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

1-Monopalmitin: 88 CA; O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

1-Monostearin: O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

Myricyl alcohol.

Myristic acid: 2807 CA; 5 DA; 49 DA; 37 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); Harple, 1952 (2–14 μ); Sinclair, 1952a (mull and CS_2 solution, 2.8–14.3 μ).

Octadeca-10,12-dienoic acid, 9,14-dihydroxy-: Davis, 1950 (mull, 2.8–13.3 μ).

Octanoic acid, 6-methyl-.

Oleic acid: 915 CA; 8,8a DA;1082 EA-Hanahan, 1952 (liquid, 2–15 μ); 1346 EA-Freeman, 1953a (liquid, 5–16 μ); 1755 EA-Ahlers, 1953 (liquid, 2.5–15μ); LB I 430-Rao, 1948 (CCl_4 solution 2–16 μ); Benedict, 1950 (liquid, 2–16 μ); French, 1954 (KI disk, 3–15 μ); Jones, 1952b (low temperature film, 7.1–8.5 μ); Jones, 1956 (p. 255, crystalline film at −196° C., 6.7–15μ); Shreve, 1950 (CS_2 solution, 2–15 μ); Sinclair, 1952b (liquid film and solid at −196° C., 3.1–19 μ); Vafiadi, 1938 (1–9 μ).

Olive oil: Freeman, 1953b (2–15 μ).

Palmitic acid: 48 CA; 6 DA; 38 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); Ard, 1951 (CS_2 solutions, with and without 1 per cent triethylamine, 2–15 μ); Harple, 1952 (2–14 μ); Shreve, 1950 (CS_2 solution, 2–15 μ); Sinclair, 1952a (mull and CS_2 solution, 2.8–14.3 μ); Swern, 1955 (CCl_4 solution, 2–12 μ; cyclohexane solution, 11.8–14.8 μ; mull, 2.4–14.4 μ); Vafiadi, 1938 (1–9 μ).

Palmitoleic acid: 1081 EA-Hanahan, 1952 (liquid, 2–15 μ).

Pelargonic acid: 60 CA; Fowler, 1953 (liquid, 6.8–13.7 μ table); Harple, 1952 (liquid, 2–14 μ).

Phrenosin: Mislow, 1952 (film from CCl_4 gel, 2–16 μ).

Phthienoic acid: 171 EA-Cason, 1951 (CS_2 solution, 5–16 μ).

Phthiocerane: Ställberg-Stenhagen, 1947 (CS_2 solution, 7.1–14.3 μ).

2-Propanol, 1-amino-2-methyl-.

Propionic acid: 1224 AA; 307 CA; 6026 CA; 53,53a DA; LB I 429-Herman, 1939 (vapor, 56° and 156° C., 1–15 μ); Barr, 1936 (liquid, 4.8–6.1 μ); Hadzi, 1953 (liquid, 6.6–20 μ; equimolar water solution, 6.6–14.3 μ); Wall, 1939 (CCl_4 solution, 2.8–5 μ).

sodium salt: 2627 EA-Childers, 1955 (mull, 7–15 μ).

Propionic acid, 3-phenyl-.

Punicic acid.

Pyruvic acid: *see* section on *Metabolites*.

Ricinelaidic acid: 1760 EA-Ahlers, 1953 (CCl_4 solution, 2.5–7.5 μ; CS_2 solution, 7.5–15 μ).

Ricinoleic acid: 1763 EA-Ahlers, 1953 (CCl_4 solution, 2.5–7.5 μ; CS_2 solution, 7.5–15 μ).

Serine, L-α-glycerylphosphoryl-.

Sorbic acid.

Sphingomyelin: Freeman, 1953b (solid film, 2–15 μ); Marinetti, 1954 (mull, 2.5–14.5 μ).

Sphingosine: 1093 EA-Mislow, 1952 ($CHCl_3$ solution, 2–16 μ).

sulfate: Mislow, 1952 ($CHCl_3$ solution, 2–16 μ).

Sphingosine, dihydro-: 1211 EA-Grob, 1952 (mull, 2.5–15 μ).

Sphingosine, *N*-lignoceryl-: Marinetti, 1954 (mull, 2.5–14.5 μ).

Stearic acid: 50 CA; 7,7a DA; 99 EA-O'Connor, 1951 ($CHCl_3$ solution, 1–12 μ); 1353 EA-Freeman, 1953a; Freeman, 1956 (CS_2 solution, 5–16 μ); 1754 EA-Ahlers, 1953 (heated liquid, 2.5–15 μ); LB I 431-Sheppard, 1945 (film, 3–14 μ); Cason, 1951 (CS_2 solution, 5–16 μ); Hadzi, 1953

(crystalline film, 6.6–20 μ); Harple, 1952 (2–14 μ); Jones, 1950 (polymorphs, mull and crystalline film, 7.1–14.3 μ); Jones, 1956 (p. 296, p. 308, β polymorph film from melt at 25° C. and at −196° C., Nujol mull of mixture of α and β polymorphs, CS_2 and CCl_4 solutions, 6.7–15.4 μ); Shreve, 1950 (CS_2 solution, 2–15 μ); Sinclair, 1952a (CS_2 solution, mulls of two polymorphic forms, and film at −196° C., 2.8–16 μ); Sinclair, 1952b (mull, 14.3–19 μ).

Stearic acid, 10-methyl-; tuberculostearic acid.

Sterculic acid.

Tariric acid.

Triacetin: Pristera, 1953 (CCl_4 solution, 2–12 μ, 14–15 μ; methylnitrate solution, 12–14 μ).

Trielaidin: 75 EA-Feuge, 1951 ($CHCl_3$ solution, 1–12 μ); O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ); Shreve, 1950 (CS_2 solution, 2–15 μ).

Trimyristin: Shreve, 1950 (CS_2 solution, 2–15 μ).

Triolein: 78 EA-Feuge, 1951 ($CHCl_3$ solution, 1–12 μ); O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ); Shreve, 1950 (CS_2 solution, 2–15 μ).

Tripalmitin: 37 CA; LB I 466-Barnes, 1944 (p. 78, film, 5.5–9.6 μ); O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

Tristearin: 86 CA; 76 EA-Feuge, 1951 ($CHCl_3$ solution, 1–12 μ); LB I 466-Barnes, 1944 (p. 78, film, 5.5–9.6 μ); O'Connor, 1955 ($CHCl_3$ solution, 2–12 μ).

Vaccenic acid: 47 DA; Ahamd, 1948 (CCl_4 solution, 12–16 μ); Rao, 1948 (CCl_4 solution, 2–16 μ).

Valeric acid: 1227 AA; 1230 AA; 304 CA; 55 DA; LB I 430-Herman, 1940 (vapor at 151° C., 1–13 μ); Hadzi, 1953 (liquid, 6.6–20 μ); *n*-Valeric acid, sodium salt: 2625 EA-Childers, 1955 (mull, 7–15 μ); Larsson, 1950 (mulls, 2–15 μ line diagrams).

Metabolites Not Otherwise Assigned

Acetaldehyde: 142 AA (gas); 5823 CA (liquid); 5824 CA (gas); LB I 426-Thompson, 1942 (gas, 1.4–20 μ); Brady, 1950 (gas, p. 501, 2–15 μ); Morris, 1943 (gas, 3.2–25 μ); Pitzer, 1949 (table and vibrational assignment).

Acetic acid (*see* section on *Lipides*).

Acetic acid, aceto-.

Acetic acid, guanidino-: 2151 CA.

Acetic acid, oxalo-.

Acetic acid, phenyl-: 1655 CA; 552 EA-Davies, 1951 (film from melt, 3–15 μ); 691 EA-Randall, 1949 (p. 106, resolidified from melt, 2–15 μ).

Acetoin.

Acetone: 1222 AA; 233 CA; 6309 CA (gas); LB I 427-Price, 1941 (gas, 3–20 μ); Barnes, 1944 (p. 81, liquid, 5.5–9.6 μ); Pierson, 1956 (gas, 2–15 μ); Pristera, 1952 (liquid, 2–15 μ); Torkington, 1945 (liquid, 3–20 μ); Wright, 1947 and 1955 (liquid, 2–15 μ).

Acetone, dihydroxy-.

Acetone phosphate, dihydroxy-.

Aceturic acid, phenyl-; phenaceturic acid:
sodium salt, hydrate: 721 EA-Randall, 1949 (p. 116, mull, 2.5–7.3 μ).
Acetyl phosphate.
cis-Aconitic acid: 766 CA.
Acrolein: 6645 CA (gas); 6646 CA; Barrow, 1953 ($CHCl_3$ solution, 1696 cm.$^{-1}$ band); Davidson, 1953 (CCl_4 solution, 971, 985, 1618 cm.$^{-1}$ bands, table).
2,4-dinitrophenyl hydrazone derivative: L. A. Jones, 1956 (KBr disk, 2–15 μ line diagram).
Adipic acid: 281 CA; LB I 431-Schönmann, 1943 (crystalline, 2.5–17μ); Corish, 1955 (crystalline in KBr disk, and melt, 2.8–15 μ); Flett, 1951 (3.8–11 μ table); Hadzi, 1953 (crystalline, 6.6–20 μ); Mann, 1948 (crystalline film, 3.1–14.3 μ polarized).
potassium salt: 6273 CA.
dipotassium salt: 5842 CA.
disodium salt; Childers, 1955 (mull, 7–15 μ).
Adipic acid, 2-amino-3-keto-.
Adipic acid, 2-keto-.
Adrenaline.
Agmatine.
Aldol: 5719 CA; Hilbert, 1936 (CCl_4 solution, 1.3–1.5 μ).
Allantoic acid.
Alloxantin.
Ammonia: 93 CA; Downie, 1953 (gas, calibration, 2–15 μ); Pierson, 1956 (gas, 2–15 μ).
Anthranilic acid: 2703 CA; 737 EA-Randall, 1949 (p. 121, mull, 2–9 μ); Ebert, 1952 (mulls of three polymorphic forms, 3–15 μ); Flett, 1951 (high temperature and normal forms, 3.8–11 μ table).
calcium salt: 4697 CA.
Anthranilic acid, 3-hydroxy-.
Auxin A; auxentriolic acid.
Auxin B; auxenolonic acid.
Azelaic acid: 142 CA; Corish, 1955 (crystalline in KBr disk and melt, 5–15 μ); Schönmann, 1943 (crystalline film, 2.7–17 μ); Wall, 1939 (CCl_4 solution, 2.8–4 μ).
dipotassium salt: 5843 CA.
Bacteriochlorophyll: Weigl, 1953 (CCl_4 solution and dried film thereof, 2.8–15.6 μ).
Benzoic acid: 779 CA; 683 EA-Randall, 1949 (p. 104, mull, 2–9 μ); 2149 EA-Anderson, 1953 (mull and KBr disk, 2–16 μ); Flett, 1951 (3.8–11 μ table); Hadzi, 1953 (crystalline film, 6.6–20 μ); Hausdorff, 1954 (KBr disk, 2–16 μ); Lacher, 1954 (in fused $SbCl_3$, 1–4 μ); Wall, 1939 (CCl_4 solution, 2.8–5 μ).
Benzoic acid, methyl ester: 710 EA-Randall, 1949 (p. 112, 3–16 μ); Rasmussen, 1949 (liquid, 2.7–7.8 μ table).
Benzoic acid, *p*-hydroxy-.
Betaine: Adams, 1949 (mull, 2.5–15.4 μ).

hydrate: 6619 CA.
hydrochloride: 5471 CA.
Bilirubin: 6555 CA; Craven, 1952 (CCl_4 solution, 2–14 μ).
Bilirubin, meso-.
Bilirubinogen, meso-.
Biliverdin.
2,3-Butanediol: 4660 CA.
Butyric acid, 2-amino,
D-, L-: Koegel, 1955 (KBr disk, 2–8 μ).
DL-: 2954 CA.
Butyric acid, 3-ethyl-2,3-dihydroxy-.
Butyric acid, 3-ethyl-2-keto-.
Butyric acid, 3-hydroxy-: 5280 CA.
Butyric acid, 3-hydroxy-2-keto-.
Butyric acid, 2,3-dihydroxy-3-methyl-.
Butyric acid, 2-keto-.
Butyric acid, 2-keto-3-methyl-.
Butyric acid, phenylamino-.
Cadaverine.
Carbamic acid, salts.
Carbon dioxide: 1924 CA; Burch, 1956 (CO_2, H_2O, N_2 analysis, 2.7 μ bands); Fowler, 1949 (respiration measurement); Howard, 1956b (gas, integrated absorption at the 1.4, 1.6, 2.0, 2.7, 4.3, 4.8, 5.2 and 15 μ bands); Osberg, 1952 (solid, 2.6–2.8 μ, 4.0–4.5 μ, 14.3–16.0 μ); Pierson, 1956 (gas, 2–15 μ); Roberts, 1951 (gas, 5 per cent in N_2, 2–6 μ).
Carbon disulfide: 698 AA; 2223 CA; Eckstein, 1954 (7–13.5 μ); Plyler, 1947 (liquid, 2–23 μ); Pristera, 1952 (liquid, 2–15 μ); Torkington, 1945 (liquid, 3–20 μ).
Catechol: 268 CA; 1923 EA-Hergert, 1953 (mull, 2.5–14.3 μ); Bard, 1955 (CH_3CN solution, 2–16 μ); Barnes, 1944 (p. 64, liquid, 5.5–9.8 μ); Davies, 1940 (solid film, 2.7–3.5 μ); Wright, 1947 and 1955 (liquid, 2–15 μ).
Chlorophyll-a: 1711 EA-Weigl, 1953 (CCl_4 solution and dried film thereof, 2.8–15.6 μ).
Chlorophyll-b: 1712 EA-Weigl, 1953 (CCl_4 solution and dried film thereof, 2.8–15.6 μ).
Choline.
Choline, acetyl-.
Choline chloride, acetyl-: 5956 CA.
Cinnamic acid: 29 CA; Flett, 1951 (3.8–11 μ table); Guy, 1949 (α and β polymorphs, 5.8–15 μ table); Mann, 1948 (crystalline film, 3.1–14.3 polarized); Randall, 1949 (p. 80, mull, 2–9 μ).
Citric acid: 765 CA; Woernley, 1952 (film from water solution, 1–15 μ).
sodium salt: 6201 CA; Sternglanz, 1956 (KBr disk and 40 per cent water solution, 2–15 μ).
Creatine: LB I 443-Klotz, 1948 (film, 5–10 μ).
monohydrate: 740 EA-Randall, 1949 (p. 122, mull, 2–9 μ).

Creatine phosphate.

Creatinine: 7198 CA; 1010 EA-Randall, 1949 (p. 230, mull, 2–9 μ); Klotz, 1948 (film, 5–10 μ).

hydrochloride: 1008 EA-Randall, 1949 (p. 229, mull, 2–9.2 μ); Mold, 1955 (Nujol and hexachlorobutadiene mulls, 3.0–3.4, 5.4–14.6 μ).

p-Cresol: 33 CA; Auméras, 1953 (CS_2 solution, 7–14 μ); Friedel, 1951 (CS_2 solution, 2.5–3.7, 7.5–15 μ); Whiffen, 1945 (CS_2 solution, 7.7–14.3 μ).

Crotonic acid: Flett, 1951 (3.8–11 μ table).

α-Cyperone: Howe, 1955 (liquid, 2.9–16 μ).

Ethanol: 426 AA; 746 AA; 1601 AA; 188 CA; 6407 CA (gas); 142, 142g DA; 397 EA-Bergstrom, 1952 (liquid, 2–15.5 μ); 929 EA-Randall, 1949 (p. 193, 2–13 μ); 1026 EA-Barrow, 1952 (vapor 25° C., 2.5–6.7 μ); LB I 424-Barnes, 1944 (p. 58, 5.5–10 μ); Laubengayer, 1954 (liquid and gas, 2–16 μ); Pierson, 1956 (gas, 2–15 μ); Pristera, 1955 (gas, 2–16 μ).

Ethylamine, indole-.

Ethylamine, phenyl-.

Ethyl mercaptan: 1112 AA; 1613 AA; 936 EA-Randall, 1949 (p. 197, liquid, 6–23 μ); Haines, 1954 (liquid 2–25 μ); Pierson, 1956 (gas, 2–15 μ); Sheppard, 1949 (gas, 2.9–14.3 μ); Sheppard, 1950 (gas, 6.7–14.3 μ); Trotter, 1946 (gas, 5.9–18.2 μ).

Formaldehyde: 1218 AA; 2538 CA; Barnes, 1944 (p. 59, 5.5–8.5 μ); Pierson, 1956 (gas, 2–15 μ); Schneider, 1956 (solid at −180°, 20° C., 2.8–3.7, 5.5–12.5 μ).

Formic acid: 1219 AA; 25 CA; 50 DA; 51 DA; 51b DA; 679 EA-Randall, 1949 (p. 103, liquid, 2–12 μ); Bonner, 1938 (vapor, various temperatures, 1–17 μ); Pierson, 1956 (gas, 2–15 μ); Williams, 1947 (gas, various temperatures, 2.5–12.5 μ).

ammonium salt: 6202 CA.

calcium salt: 6636 CA.

Fumaric acid: 472 CA; Flett, 1951 (3.8–11 μ table); Murray, 1954 (mull, 3 μ, 6 μ, bands).

Glutaraldehyde, 2-keto-.

Glutaric acid: 7386 CA; 686 EA-Randall, 1949 (p. 104, mull, 2–9 μ); LB I 432 (line diagram)-Schönmann, 1943 (crystalline at 20° C. and melt at 105° C., 2.7–17 μ); Hadzi, 1953 (crystalline, 6.6–20 μ); Wehrli, 1941 (crystalline, 2.5–16 μ).

α and β polymorphic forms: Corish, 1955 (crystalline in KBr disks and molten, 5–15 μ).

disodium salt: Childers, 1955 (mull, 7–15 μ).

Glutaric acid, 2-aceto-.

Glutaric acid, 2-keto-.

Glycol, ethylene-: 1602 AA; 2166 CA.

Glycolic acid: 5829 CA; Flett, 1951 (3.7–11 μ table).

Glyoxal, methyl-.

Glyoxylic acid.

Gossypol: 2347 EA-O'Connor, 1954a ($CHCl_3$ solution, 2–12 μ).

Hematinic acid, carboxylated-: MacDonald, 1952 (mull, 3–15.5 μ).
Hemin I, etio-: Vestling, 1939 (CCl_4 solution, 2.8–4 μ).
cis-Hex-3-en-1-ol; leaf alcohol: Crombie, 1950 (5–15 μ).
Hippuric acid: 2802 CA; 775 EA-Randall, 1949 (p. 135, mull, 2–9 μ); Bellamy, 1954 (p. 242, 5.5–15.4 μ).
sodium salt, hydrate: 720 EA-Randall, 1949 (p. 116, mull, 2–9 μ).
Histamine: 5860 CA.
hydrochloride: 5455 CA.
Histamine acid phosphate.
Homogentisic acid.
Hydantoic acid: 8334 CA.
Hydantoin: 872 EA-Randall, 1949 (p. 173, mull, 2–15 μ).
Imidazolacetic acid.
Imidazole-5-carboxamide, 4-amino-.
Imidazolformic acid.
Imidazolpropionic acid.
Indican.
Indole-3-acetic acid: 670 CA.
Indole-3-acetic acid, 5-hydroxy-.
Indole-3-propionic acid.
2-Indolylacetaldehyde: J. B. Brown, 1952 (2.8–14.3 μ).
Indoxol.
Indoxylglucuronic acid.
α-Ionone: Naves, 1953 (vaseline mull, 5.5–14.3 μ line diagram).
Isobutyric acid, 3-amino-.
Isocaproic acid, 2-hydroxy-.
Isocaproic acid, 2-keto-.
Isocitric acid.
Isovaleric acid, 2-hydroxy-.
Khellin; 2-methyl-5,8-dimethyoxy-furanochromone: Bailey, 1951 ($CHCl_3$ solution, 2–12.5 μ).
Kynurenic acid; 4-hydroxyquinoline-2-carboxylic acid.
Kynurenine; 3-*o*-aminobenzolylalanine: Warnell, 1954 (mull, 3 μ, 6 μ, bands).
Kynurenine, formyl-.
Kynurenine, 3-hydroxy-.
Lactic acid: 5338 CA; 1164 EA-J. T. Barr, 1952 (2–15 μ); Flett, 1951 (3.7–11 μ table).
Lactic acid, 2-aceto-.
Lavandulol: W. Kuhn, 1952 (2–16 μ).
Levulinic acid, 5-amino-.
d-Limonene: 1142 EA-Bain, 1952 (liquid, 2–16 μ); 2202 EA-O'Connor, 1954b ($CHCl_3$ solution, 2–12 μ).
α-Lipoic acid; 6,8-dithio-*n*-octanoic acid: 2082 EA-Hornberger, 1953 (CCl_4 solution, 2.8–12 μ).
Lupulone: Howard, 1952 (mull, 2–10 μ).
Lysergic acid: Kornfeld, 1956 (mull, 2.6–12 μ).

Maleic acid: 495 CA; 6253 CA; Cardwell, 1953 (mull, 2–8.5 μ); Flett, 1951 (3.8–11 μ table); Murray, 1954 (mull, 3 μ, 6 μ bands); Smith, 1956 (2–16 μ line diagram).
anhydride: 1870 EA-Bickford, 1953 ($CHCl_3$ solution, 1–12 μ).
dipotassium salt: 6203 CA.
disodium salt: 8103 CA.
Malic acid: 504 CA; 7452 CA.
Malonic acid: 2960 CA; LB I 432-Schönmann, 1943 (2.7–17 μ line diagram); Flett, 1951 (3.8–11 μ table); Hadzi, 1953 (crystalline, 6.6–20 μ).
potassium salt: 6204 CA.
disodium salt: 2629 EA-Childers, 1955 (mull, 7–15 μ).
Mandelic acid; 2-hydroxy-2-phenylacetic acid: 477 CA; Flett, 1951 (3.8–11 μ table).
potassium salt: 6205 CA.
Methane: 97 AA; 528 AA; 4063 CA; LB I 361-Coblentz, 1905; Pierson, 1956 (gas, 2–15 μ).
Methanol: 425 AA; 745 AA; 1918 CA; 1919 CA (vapor); 924 EA-Randall, 1949 (p. 191, 2–12 μ); LB I 424-Barnes, 1944 (p. 58, 5.5–10 μ); Pierson, 1956 (gas, 2–15 μ); Smith, 1951 (liquid and CCl_4 and CS_2 solutions, 2–15 μ); Torkington, 1945 (3–20 μ).
Methyl mercaptan; methylthiol: Pierson, 1956 (gas, 2–15 μ); Thompson, 1940 (gas, 1.4–16 μ); Trotter, 1946 (gas, 5.9–18.2 μ).
Neoherculin: Crombie, 1952 (mull, 2.8–14 μ); Crombie, 1955 (discussion).
Nicotine: 2193 EA-Eddy, 1954 (liquid, and CCl_4 and acetone solutions, 2–15 μ); Witkop, 1954b (liquid, 2.5–7.0 μ).
mono and dihydrochloride: Witkop, 1954b ($CHCl_3$ solutions, 2.5–7.0 μ).
Noradrenaline.
Oxalic acid: 2962 CA; 689 EA-Randall, 1949 (p. 105, mull, 2–9 μ); Duval, 1941 (polymorphism? 6.5–14 μ table).
anhydrous: Hadzi, 1953 (sublimed film, 6.6–14.7 μ).
dihydrate: LB I 431-Fichter, 1940 (film, 2.6–14.3 μ); LB I 432-Schönmann, 1943 (crystalline, 2.7–17 μ line diagram).
calcium salt, anhydrous, monohydrate and three other hydrates: Lecomte, 1943 (6–13 μ table).
disodium salt: 2630 EA-Childers, 1955 (mull, 7–15 μ).
Pelargonic acid: 60 CA.
Phenol: 1213 AA; 1612 AA; 843 CA; 2227 EA-Davies, 1954 (CCl_4 solution, 7.1–11.2 μ); 2358 EA-O'Connor, 1954a ($CHCl_3$ solution, 2–12 μ); Barnes, 1944 (p. 61, 5.5–9.6 μ); Lacher, 1954 (in fused $SbCl_3$, 1–4 μ); Whiffen, 1945 (CS_2 solution, 7.7–14.3 μ).
Phenolsulfuric acid.
Phloroglucinol; 1,3,5-trihydroxybenzene: 3845 CA.
dihydrate: 2365 EA-O'Connor, 1954a (mull, 2–12 μ).
Phthalic acid; benzene-*o*-dicarboxylic acid: 6272 CA; Cardwell, 1953 (mull, 2–8.5 μ); Flett, 1951 (4–11 μ table); Wright, 1955 (mull, $Pb(SCN)_2$ as internal standard, 2.5–16 μ).
anhydride: Randall, 1949 (p. 79, mull, 2–9.2 μ).

Phthalocyanines: Ebert, 1952 (mulls, polymorphism, 3–15 μ).
Picrotoxin and related compounds: Conroy, 1952 (mulls, 2–16 μ).
Pimelic acid; pentane-1,5-dicarboxylic acid; 1,7-heptanedioic acid: 5961 CA; LB I 432 (line diagram)-Schönmann, 1943 (crystalline, 2.7–17 μ); Corish, 1955 (crystalline in KBr disk and melt, 5–15 μ).
disodium salt: 2632 EA-Childers, 1955 (mull, 7–15 μ).
α-Pinene: 1137 EA-Bain, 1952 (liquid, 2–16 μ); 2200 EA-O'Connor, 1954b ($CHCl_3$ solution, 2–12 μ); Barnes, 1944 (p. 100, liquid, 5.9–9.5 μ).
β-Pinene: 2188 CA; 1138 EA-Bain, 1952 (liquid, 2–16 μ); 2201 EA-O'Connor, 1954b ($CHCl_3$ solution, 2–12 μ); Barnes, 1944 (p. 100, liquid 5.5–9.6 μ).
Pipecolic acid; hexahydropicolinic acid:
hydrochloride: Zacharius, 1954 (mull, 2–15 μ).
Porphyrin, copro-,
I and III: Craven, 1952 (CCl_4 solutions, 2–14 μ).
Porphyrin, deutero-: Craven, 1952 (CCl_4 solution, 2–14 μ).
Porphyrin I, etio-: Vestling, 1939 (CCl_4 solution, 2.8–4 μ).
Porphyrin, hemato-.
Porphyrin I, meso-.
Porphyrin, proto-: Craven, 1952 (CCl_4 solution, 2–14 μ).
Porphyrin, uro-.
Pteridine derivatives: Mason, 1955 (KBr disks, spectra and table).
Putrescine; 1,4-diamino-*n*-butane.
Pyrogallol; 1,2,3-trihydroxybenzene: Wulf, 1936 (CCl_4 solution, 1.4–1.5 μ).
Pyruvic acid; 2-oxopropanoic acid: 5963 CA; 685 EA-Randall, 1949 (p. 104, liquid, 2–9 μ); 2337 EA-Thomas, 1955 (2–15.3 μ).
Pyruvic acid, 2-phosphoenol-.
Quinolinic acid: 5964 CA.
Resorcinol; 1,3-dihydroxybenzene: 467 CA; Bard, 1955 (CH_3CN solution, 2–16 μ), Barnes, 1944 (p. 64, film, 5.5–9.6 μ); Davies, 1940 (solid film, 2.7–3.5 μ).
Serotonin; 5-hydroxytryptamine; creatine sulfate monohydrate derivative: Speeter, 1951 (mull, 2.8–14.3 μ).
Skatole; 3-methylindole: 3823 CA; Geissman, 1952 (2–10 μ).
Spermidine.
Spermine.
Squalene: 1144 EA-Dauben, 1952a (liquid, 2–16 μ); 1103 EA-Dauben, 1952b (liquid, 2–16 μ); Thompson, 1945 (5.6–16 μ).
Stercobilin.
Stercobilinogen.
Succinamide: Chouteau, 1953 (mull, double pass, CaF_2 prism, 2–8.5 μ).
Succinic acid; butanedioic acid: 2805 CA; 687 EA-Randall, 1949 (p. 105, mull, 2–9 μ); Flett, 1951 (3.8–11 μ table); Hadzi, 1953 (crystalline, 6.6–20 μ); Koegel, 1955 (KBr disk, 2–8 μ table); Schönmann, 1943 (crystalline, line diagram 2.7–17 μ).
β-polymorph: Corish, 1955 (crystalline in KBr disk and melt, 5–15 μ).
ammonium salt: 1834 CA.

dipotassium salt: 1837 CA.
disodium salt: 2628 EA-Childers, 1955 (mull, 7–15 μ).
Succinic acid, oxalo-.
Succinic acid, 2-ureido-.
Tartaric acid; 2,3-dihydroxybutane-1,4-dioic acid: 658 CA.
ammonium salt: 495 CA.
calcium salt: 6638 CA.
potassium salt: 480 CA.
Taurine; 2-aminoethanesulfonic acid.
α-Terpinene; *p*-menthadiene-1,3: O'Connor, 1954b ($CHCl_3$ solution, 2–12 μ).
γ-Terpinene; *p*-menthadiene-1,4: O'Connor, 1954b ($CHCl_3$ solution, 2–12 μ).
Tetronic acid and derivatives; lactones of 3-hydroxy-2-keto acids: Duncanson, 1953 (mulls and solutions, 5.4–6.7 μ).
Tryptamine, 5-hydroxy-.
Tyramine.
Urea: 447 CA; 862 EA-Randall, 1949 (p. 169, mull, 2–9 μ); Barnes, 1944 (p. 88, 5.7–8.3 μ); Clark, 1952a (film from ethanol solution, 2–16 μ); Keller, 1948 (crystal, polarized bands at 1680, 1590, 1160, 1010, and 546 $cm.^{-1}$); Kellner, 1941 (quartz prism, crystalline deposit, 2.8–3.2 μ); Krimm, 1955 (solid, 1680 $cm.^{-1}$ band, CO..HN = 180°); Margoshes, 1954 (C=O, 1.26 Å., 1665 $cm.^{-1}$); Verne, 1955 (dry powders and vaseline mull, 5–16.7 μ); Waldron, 1950 (3342, 3362, 3436, 3449 $cm.^{-1}$ bands, polarization studies).
Urethane; ethylcarbamate: 828 EA-Randall, 1949 (p. 157, 2.6–9 μ).
Urocanic acid; iminazolylacrylic acid.
Valeric acid, 2-keto-.
Valeric acid, 2-keto-3-methyl-.
Water: 1019 EA-Randall, 1949 (p. 234, liquid, 2–15 μ); Adams, 1956 (liquid, 4 μ thickness, 2.5–15.4 μ, band centers at 3375 and 1640 $cm.^{-1}$); Barr, 1940 (liquid, 1.8–4.6 μ); Curcio, 1951 (liquid, 0.7–2.5 μ); Downie, 1953 (vapor, calibration, 2–35 μ); Freeman, 1956-Blout, 1953 (liquid, 5–16 μ); Gore, 1949 (liquid, 2.5–15.4 μ); Howard, 1956a (vapor, 2.3–3.6 μ); Howard, 1956c (vapor, integrated absorption, 0.95, 1.1, 1.38, 1.87, 2.7, 3.2 and 6.3 μ bands); Mills, 1955 (vapor, CsI prism, 23–50 μ); Nielsen, 1950 (vapor, 5.88–6.63 μ); Oetjen, 1952 (vapor, grating, 42–160 μ); Plyler, 1953 (vapor, CsI prism, 37–52 μ); Plyler, 1954 (liquid, 2–42 μ); Sternglanz, 1956 (liquid, 2–15 μ); Williams, 1937 (liquid, 2–8 μ); Wright, 1955 (liquid, 2.5–11 μ; BaF_2 for cell windows).
Xanthurenic acid.

Nucleic Acid Derivatives, with up to Two Atoms Substituted

Adenine: 7006 CA; LB II 652-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).
hydrochloride: Bentley, 1951 (ethanol solution, 3.4–4.5 μ; mull, 5.3–14.3 μ); Clark, 1950a (mull, 2–16 μ).
sulfate: 952 EA-Randall, 1949 (p. 209, mull, 2–15 μ); Barnes, 1944 (mull, 5–13 μ); Clark, 1950a (mull, 2–16 μ).

Adenosine: 5446 CA; LB II 653-Blout, 1949 (evaporated film and powder, 2–15 μ); Clark, 1950a (mull, 2–16 μ); Clark, 1952 (mull, 2–16 μ).

Adenosine, deoxy-.

Adenosine diphosphate.

Adenosine triphosphate: 5472 CA.

dibarium salt: Schwarz, 1952 (mull, 8.5–10.5 μ table).

disodium salt: 6515 CA.

Adenylic acid, yeast-; 2′ and 3′ adenylic acids: LB II 653-Blout, 1949 (powder, 5–15 μ); Blout, 1948 (powder, 5.6–14.3 μ line diagram); Clark, 1950a (mull, 2–16 μ); Dekker, 1954 (mull, 2.5–14.7 μ).

Adenylic acid a; adenosine-2′-phosphate: D. M. Brown, 1952 (mull, 2–15.5 μ).

Adenylic acid b; adenosine-3′-phosphate: D. M. Brown, 1952 (mull, 2–15.5 μ).

Adenylic acid, muscle-; adenosine-5′-phosphate: 5473 CA; D. M. Brown, 1952 (mull, 2–15.5 μ); Clark, 1950a (mull, 2–16 μ); Lenormant, 1954 (dry and D_2O solutions, various *p*H, 5.7–11.1 μ).

Adenylic acid, deoxy-; 2′-deoxy-adenosine-3′ or 5′-phosphate.

Allantoin: 6488 CA; 874 EA-Randall, 1949 (p. 174, mull, 2–9 μ); Clark, 1950a (mull, 2–16 μ).

Caffeine: 1036 CA; 2500 CA; 147 EA-Parke, 1951 (analysis); 953 EA-Randall, 1949 (p. 210, mull, 2–9 μ); LB II 654-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Crotonoside; isoguanine-D-riboside; β-D-ribofuranosylisoguanine: Clark, 1950a (mull, 2–16 μ); Davoll, 1951 (mull, 2–16 μ).

Cytidine: Clark, 1950a (mull, 2–16 μ); Woernley, 1952 (film from water suspension, 1–15 μ).

Cytidine-5′-phosphate: 2222 EA-Michelson, 1954 (5.5–14.3 μ); Harris, 1953 (paraffin and "fluorocarbon" mulls, 2.7–15.4 μ).

Cytidine, deoxy-.

Cytidine-3′-phosphate, deoxy-: 2224 EA-Michelson, 1954 (5.5–14.3 μ).

Cytidine-5′-phosphate, deoxy-: 2223 EA-Michelson, 1954 (5.5–14.3 μ).

Cytidine-5′-phosphate, 5,6-dihydro-.

Cytidylic acid: LB II 653-Blout, 1949 (powder, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Cytidylic acid a; cytidine-2′-phosphate?

two crystalline forms: Harris, 1953 (paraffin and fluorocarbon mulls, 2.7–15.4 μ);

polymorphic form a: 2225 EA-Michelson, 1954 (5.5–14.3 μ).

Cytidylic acid b; cytidine-3′-phosphate?: 2225 EA-Michelson, 1954 (5.5–14.3 μ); Harris, 1953 (paraffin and fluorocarbon mulls, 2.7–15.4 μ).

Cytidylic acid, deoxy-; 2′-deoxy-cytidine-3′-phosphate: 2224 EA-Michelson, 1954 (5.5–14.3 μ).

Cytosine: 610 EA-Short, 1952 (No. 59, mull, 2–25 μ); LB II 652-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ; possible polymorphism); Stimson, 1952 (KBr disk, 2.5–14.5 μ).

hydrate: Blout, 1951 (mull and single crystal, 2–15 μ polarized).

Cytosine deoxyriboside hydrochloride: Dekker, 1951 (mull, 2.5–15 μ).

Cytosine, 5-methyl-; 4-amino-5-methyl-2-pyrimidone: Clark, 1950a (mull, 2–16 μ).

Cytosine, 5-methyl-, deoxyriboside hydrochloride: Dekker, 1951 (mull, 2.5–15 μ).

Guanine: 427, 428 EA-Scott, 1952 (sublimed film, 3–12 μ); LB II 652-Blout, 1950b (evaporated film and powder, 2–15 μ); Clark, 1950a (mull, 2–16 μ); Woernley, 1952 (film from water suspension, 1–15 μ).

Guanosine: LB II 652-Blout, 1949 (mull, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Guanosine, deoxy-.

Guanylic acid: LB II 653-Blout, 1949 (cast film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Guanylic acid, deoxy-.

Hypoxanthine: Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Inosine.

Inosine diphosphate.

Inosine triphosphate.

Inosinic acid.

Orotic acid: 6467 CA; Clark, 1950a (mull, 2–16 μ).

Purine: Beaman, 1954 (mull, 2–16 μ).

Purine, 2,6-diamino-.

2,8-Purinedione, 6-amino-.

Theobromine; 3,7-dimethyl-2,6(1,3)-purinedione: LB II 654-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Theophylline; 1,3-dimethylxanthine; 1,3-dimethyl-2,6(1,3) purinedione: 493 CA; LB II 654-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Thymidine.

Thymidylic acid.

Thymine: LB II 652-Blout, 1950b (evaporated film, 2–15 μ); Blout, 1950a (sublimed film); Clark, 1950a (mull, 2–16 μ); Woernley, 1952 (film from alcohol suspension, 1–15 μ).

Uracil: 6335 CA; 597 EA-Short, 1952 (No. 41, mull, 2–25 μ); 881 EA-Randall, 1949 (p. 176, mull, 2–9 μ); LB II 652-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ); Thompson, 1950 (perfluorokerosene and paraffin mulls, 2.8–3.1 μ, 5.6–14.5 μ line diagram).

Uric acid: 2963 CA; 502 DA; 950 EA-Randall, 1949 (p. 208, mull, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Uridine: Clark, 1950a (mull, 2–16 μ).

Uridine-5′-phosphate.

Uridine-5′-pyrophosphate, monobarium salt: 1503 EA; 2595 EA-Anand, 1952 (mull, 2–15.5 μ).

Uridine triphosphate.

Uridylic acid: Clark, 1950a (mull, 2–16 μ).

Xanthine: 654 CA; 951 EA-Randall, 1949 (p. 209, mull, 2–15 μ); LB II 652-Blout, 1950b (evaporated film, 2–15 μ); Clark, 1950a (mull, 2–16 μ).

Xanthosine: Blout, 1949 (mull, 2 15 μ); Clark, 1950a (mull, 2 16 μ).
Xanthylic acid.

Steroids

Adrenosterone; *see* 4-Androstene-3,11,17-trione.
Aldosterone; *see* 11β,21-dihydroxy-3,20-diketo-4-Pregnen-18-al.
Allocholesterol; *see* 4-Coprosten-3β-ol.
Allopregnane-3α,20α-diol.
Allopregnane-3β,20α-diol: Dobriner, 1953 (No. 74, $CHCl_3$ solution, 5.5 6.1, 8.7–11.8 μ).
Allopregnane-3β,20β-diol, diacetate: Dobriner, 1953 (No. 76, CS_2 solution, 5.6–6.1, 7.1–11.4 μ; CCl_4 solution, 6.7–7.7 μ).
Allopregnane-3β,17α,20α-triol, 3,20-diacetate: Dobriner, 1953 (No. 84, $CHCl_3$ solution, 5.5–6.1, 8.7–11.8 μ).
Allopregnane-3β,17α,20β-triol, 3,20-diacetate: Dobriner, 1953 (No. 85, $CHCl_3$ solution, 5.5–6.1, 8.7–11.8 μ).
Allopregnane-3β,17α,20β,21-tetrol, 3,20,21-triacetate: Dobriner, 1953 (No. 90, 5.5–6.1, 8.7–11.8 μ).
Allopregnane-3β,11β,17α,20β,21-pentol: Rosenkrantz, 1955a (film, 10.0-10.8 μ table).
Allopregnane-3,20-dione: Dobriner, 1953 (No. 117, CS_2 solution, 5.6-6.1, 7.4-14.3 μ; CCl_4 solution, 6.7 7.5 μ); Furchgott, 1946b (crystalline film from melt, 2–12.4 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).
Allopregnane-11,20-dione, 3β,17α,21-trihydroxy-: Rosenkrantz, 1955a (film, 10.0–10.8 μ table).
Allopregnan-20-one, 3α-hydroxy-: Jones, 1949 (CS_2 solution, 8.4–11.2 μ).
Allopregnan-20-one, 3β-hydroxy-: 2433 EA-Dobriner, 1953 (No. 150, CS_2 solution, 5.6 6.0, 7.2 11.2 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).
Allopregnan-20-one, 3β,11β,21-trihydroxy-: Rosenkrantz, 1955a (film, 10.0 10.8 μ table).
Allopregnan-20-one, 3β,17α,21-trihydroxy-: Rosenkrantz, 1955a (film, 10.0-10.8 μ table).
Allopregnan-20-one, 3α,11β,17α,21-tetrahydroxy-, 3,21-diacetate: 2470 EA-Dobriner, 1953 (No. 187, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ).
Allopregnan-20-one, 3β,11β,17α,21-tetrahydroxy-: Chamberlin, 1955 (3 μ, 6 μ band maxima); Rosenkrantz, 1955a (film, 10.0 10.8 μ table).
3,5-Androstadien-17-one: Jones, 1949 (CS_2 solution, 8.4 11.2 μ).
Androstane-3α,17β-diol: Dobriner, 1953 (No. 65, $CHCl_3$ solution, 5.6 6.2, 8.7–11.8 μ).
Androstane-3α,16α,17β-triol, triacetate: Dobriner, 1953 (No. 81, CS_2 solution, 5.6–6.0, 7.6–11.5 μ).
Androstane-3,17-dione: Dobriner, 1953 (No. 110, CS_2 solution, 5.6 6.1, 7.4 12.5 μ; CCl_4 solution, 6.7 7.6 μ); Furchgott, 1946a (glassy film from pyridine, 2 12.3 μ); Jones, 1949 (CS_2 solution, 8.4 11.2 μ); Jones, 1955b (CCl_4 solution, 7.0–7.7 μ; CS_2 solution, 7.7–14.3 μ).

Androstane-3,11,17-trione: Dobriner, 1953 (No. 122, CS_2 solution, 5.6–6.0, 7.2–14.1 μ; CCl_4 solution, 6.7–7.6 μ).

Androstane-3,17-dione, 11β-hydroxy-.

Androstan-11,17-dione, 3α-hydroxy-; 11-keto-androsterone: 2478 EA-Dobriner, 1953 (No. 195, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

Androstan-17-one, 3α-hydroxy-; androsterone: 718 CA; LB I 464-Dobriner, 1948a (CS_2 solution, 2.6–18.2 μ); Blout, 1950a (CS_2 solution); Blout, 1951 (mull and single crystal, 2–15 μ); Blout, 1955 (microcrystal, 5–15 μ); Cole, 1952b (CS_2 solution, microscope, 7.4–14.8 μ); Cole, 1954 (solutions, 2.7–14.3 μ); Dobriner, 1953 (No. 135, CS_2 solution, 5.6–6.0, 7.2–12.8 μ; No. 295, CS_2 solution, 2.7–3.6 μ); Furchgott, 1946a (crystalline film from pyridine, 2–12.3 μ); Jones, 1948a (CS_2 solution, 2.7–4.0, 5.4–6.0, 7.0–12.5 μ)-Dobriner, 1948b; Jones, 1948b (CS_2 solution, 2–13 μ); Jones, 1949 (CS_2 solution, 2–13 μ); Jones, 1955a (CS_2 solution, 7.2–14 μ table).

Androstan-17-one, 3β-hydroxy-; epiandrosterone; isoandrosterone: LB I 465-Dobriner, 1948a (CS_2 solution, 2.6–18.2 μ); Cole, 1952a (CS_2 solution, 7.4–14.3 μ); Cole, 1954 (solutions, 2.7–14.3 μ); Dobriner, 1953 (No. 137, CS_2 solution, 5.6–5.9, 7.4–12.8 μ; No. 296, CCl_4 solution, 2.7–3.6 μ); Furchgott, 1946a (crystalline film from pyridine, 2–12.3 μ); Jones, 1948b (CS_2 solution, 6.8–11.2 μ); Jones, 1949 (CS_2 solution, 6.8–11.2 μ); Rosenkrantz, 1955b (p. 13, CS_2 solution, 7.2–11.5 μ).

Androstan-17-one, 3α,11β-dihydroxy-; 11β-hydroxyandrosterone: 2448 EA-Dobriner (No. 165, $CHCl_3$ solution, 1736 cm.$^{-1}$, 8.7–11 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

4-Androstene-3,17-dione: 2137 EA-2138 EA-2139 EA-Rosenkrantz, 1953b (mull, melt, and CS_2 solution, 7.5–12.5 μ); Dobriner, 1953 (No. 112, CS_2 solution, 5.6–6.1, 7.2–14.3 μ; CCl_4 solution, 6.7–7.6 μ; No. 113, $CHCl_3$ solution, 5.6–6.3, 8.7–11.8 μ; No. 300, CCl_4 solution, 2.1–3.6 μ); Furchgott, 1946a (crystalline film from pyridine, 2–12.3 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ); Jones, 1955a (CS_2 solution, 7.2–14.3 μ).

4-Androstene-3,17-dione, 11β-hydroxy-: 2475 EA-Dobriner, 1953 (No. 192, $CHCl_3$ solution, 5.6–6.3, 8.7–11.8 μ).

4-Androstene-3,11,17-trione; adrenosterone: 718 CA; Dobriner, 1953 (No. 123, $CHCl_3$ solution, 5.5–6.3, 8.7–11.8 μ); Furchgott, 1947b (crystalline film from melt, 2–12.4 μ); Rosenkrantz, 1955b (CS_2 solution, KBr disk, film, and mull, 7.5–10.8 μ).

5-Androstene-3β,17β-diol: Dobriner, 1953 (No. 69, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ).

5-Androstene-3β,16α,17β-triol, triacetate: Dobriner, 1953 (No. 82, CS_2 solution, 5.6–6.1, 7.2–12.7 μ; CCl_4 solution, 6.7–7.6 μ).

16-Androsten-3α-ol: Dobriner, 1953 (No. 28, CCl_4 solution, 6.7–7.6 μ; CS_2 solution, 7.4–14.3 μ); Rosenkrantz, 1953a (CS_2 solution, 7.5–11 μ).

4-Androsten-3-one, 17α-hydroxy-; epitestosterone: Jones, 1955a (CS_2 solution, 7.2–14 μ table); Rosenkrantz, 1953a (CS_2 solution, 7.5–11 μ).

4-Androsten-3-one, 17β-hydroxy-; testosterone: 727 CA; Dobriner, 1953 (No. 130, CS_2 solution, 5.7–6.1, 7.2–13.7 μ; CCl_4 solution, 6.7–7.7 μ; Furchgott, 1946a (crystalline film from pyridine, 2 12.3 μ); Jones, 1949 (CS_2 solution, 8.4 11.2 μ); Jones, 1955a (CS_2 solution, 7.2 14 μ table).

5-Androsten-17-one, 3β-chloro-; Dobriner, 1953 (No. 96, CS_2 solution, 5.6 6.1, 7.2–14.3 μ; CCl_4 solution, 6.7–7.6 μ); Furchgott, 1946a (crystalline film from pyridine, 2 12.3 μ); Jones, 1949 (CS_2 solution, 8.4 11.2 μ).

5-Androsten-17-one, 3β-hydroxy-; dehydroepiandrosterone; dehydroisoandrosterone: Dobriner, 1953 (No. 139, CS_2 solution, 5.6–6.1, 7.2–12.8 μ; CCl_4 solution, 6.7–7.6 μ); Furchgott, 1946a (crystalline film from pyridine, 2–12.3 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ); Jones, 1955a (CS_2 solution,7.2 14 μ table); White, 1950 ($CHCl_3$ solution, 2–13 μ).

9-Androsten-17-one, 3α-hydroxy-: Jones, 1949 (CS_2 solution, 8.4-11.2 μ).

Androsterone; *see* 3α-hydroxy-Androstan-17-one.

Androsterone, 11β-hydroxy-; *see* 3α,11β-dihydroxy-Androstan-17-one.

Androsterone, 11-keto-; *see* 3α-hydroxy-Androstane-11,17-dione.

Calciferol; D_2 vitamin: Dobriner, 1953 (No. 56, $CHCl_3$ solution, 5.6 6.3 μ; CCl_4 solution, 6.7–7.6 μ; CS_2 solution, 7.2–14.3 μ); Jones, 1950 (CCl_4 solution, 6.8–7.9 μ; CS_2 solution, 7.9–14.3 μ); Pirlot, 1948 (CS_2 solution, 8–12 μ); Rosenkrantz, 1952a (crystalline film, 2–13 μ); Turnbull, 1950 (mull, 7.7–15.4 μ).

Cholanic acid, 3α,7α-dihydroxy-; chenodeoxycholic acid: methyl ester: 2524 EA-Dobriner, 1953 (No. 241, CS_2 solution, 5.6–6.1, 7.2–14.3 μ; CCl_4 solution, 6.7–7.6 μ); Wooton, 1953 (CS_2 solution, 8.2–11.1 μ).

Cholanic acid, 3α,12α-dihydroxy-; deoxycholic acid: 2525 EA-Dobriner, 1953 (No. 242, CS_2 solution, 5.6–6.1, 7.2–11.4 μ; CCl_4 solution, 6.7–7.6 μ).
methyl ester: Wooton, 1953 (CS_2 solution, 8.2–11.1 μ).

Cholanic acid, 3α,7α,12α-trihydroxy-; cholic acid: 722 CA; 5428 CA.
methyl ester: Wooton, 1953 (CS_2 solution, 8.2–11.1 μ).

5,7-Cholestadien-3β-ol; 7-dehydrocholesterol: 1378 EA-D. R. Johnson, 1953 (film from melt, 2 16 μ); Dobriner, 1953 (No. 45, CCl_4 solution, 6.7 7.6 μ; CS_2 solution, 7.2–15.4 μ).

Cholestan-3β-ol; cholestanol; dihydrocholesterol: 501 EA-Fürst, 952 (CS_2 solution, 2–14.5 μ); 1371 EA-D. R. Johnson, 1953 (film from melt, 2 16 μ); Bladon, 1951 (CCl_4 solution, 3 μ, 6 μ regions; CS_2 solution, 10–15 μ); Dobriner, 1953 (No. 40, CS_2 solution, 5.6–6.1, 7.2–14.1 μ; CCl_4 solution, 6.7 7.6 μ); Lieberman, 1948 (CS_2 solution, 8.4 11.4 μ); Rosenkrantz, 1952b (film from melt, 2 16 μ); Rosenkrantz, 1955b (film, 2–13 μ); Woodward, 1952 (CS_2 solution, 2–12 μ).

5-Cholesten-3β-ol; cholesterol: 723 CA; 7247 CA; 1377 EA-D. R. Johnson, 1953 (film from melt, 2 16 μ); LB I 465-Baird, 1947 (CS_2 solution, 2 16 μ); Barer, 1949 (crystal, 2.6 3.6 μ, 6.3 12.5 μ); Bladon, 1951 (3 μ, 6 μ regions in CCl_4; mull and CS_2 solution, 10 15 μ); Blout, 1952b (CCl_4 solution, 2.3 μg. solute, 5.9 12.5 μ); Clark, 1955 (CCl_4 and CS_2 solutions, 1–15.3 μ); Coates, 1953 (CS_2 solution, microgram sample, 2.5 11 μ); Dobriner, 1953 (No. 41, CCl_4 solution, 5.6 6.3, 6.7 7.5 μ;

CS_2 solution, 7.2–15.4 μ); Fontaine, 1951 (CCl_4 solution, 2–7.5 μ; CS_2 solution, 7.5–15 μ); Freeman, 1953b (CS_2 solution, 2–14 μ); Jones, 1950 (crystalline film, 7.1–14.3 μ); Rosenkrantz, 1952b (film from melt, 2–16 μ).

7-Cholesten-3β-ol: 1375 EA-D. R. Johnson, 1953 (film from melt, 2–16 μ); Dobriner, 1953 (No. 44, CS_2 solution, 5.6–6.2, 7.2–15.4 μ); Idler, 1952 ($CHCl_3$ solution, 2–11 μ).

4-Cholesten-3-one: 215 EA-Josien, 1951 (mull, 3–12 μ line diagram); Dobriner, 1953 (No. 103, CCl_4 solution, 5.8–6.3, 6.6–7.6 μ; CS_2 solution, 7.2–15.4 μ); Jones, 1955a (CS_2 solution, 7.2–14 μ table); Rosenkrantz, 1952b (solid from melt, 2–16 μ).

Cholesterol; *see* 5-Cholesten-3β-ol.

Cholesterol, 7-dehydro-; *see* 5,7-Cholestadien-3β-ol.

Cholic acid; *see* 3α,7α,12α-trihydroxyCholanic acid.

Coprostan-3β-ol: Cole, 1952a (CS_2 solution, 7.4–14.3 μ); Dobriner, 1953 (No. 50, CS_2 solution, 7.4–12.5 μ); Rosenkrantz, 1955a (CS_2 solution, 10.0–10.8 μ table); Rosenkrantz, 1955b (film, 2–13 μ).

4-Coprosten-3β-ol.

Corticosterone; *see* 11β,21-dihydroxy-4-Pregnene-3,20-dione.

Corticosterone, 11-dehydro-; *see* 21-hydroxy-4-Pregnene-3,11,20-trione.

Corticosterone, 11-dehydro-17-hydroxy-; *see* 17α,21-dihydroxy-4-Pregnene-3,11,20-trione.

Corticosterone, 11-deoxy-17-hydroxy-; *see* 17α,21-dihydroxy-4-Pregnene-3,20-dione.

Corticosterone, deoxy-; *see* 21-hydroxy-4-Pregnene-3,20-dione.

Corticosterone, 17-hydroxy-; *see* 11β,17α,21-trihydroxy-4-Pregnene-3,20-dione.

Cortisol; *see* 11β,17α,21-trihydroxy-4-Pregnene-3,20-dione.

Cortisone; *see* 17α,21-dihydroxy-4-Pregnene-3,11,20-trione.

Cortisone, hydro-; *see* 11β,17α,21-trihydroxy-4-Pregnene-3,20-dione.

Cortisone, tetrahydro-; *see* 3α,17α,21-trihydroxyPregnane-11,20-dione.

D_2 vitamin; *see* Calciferol.

D_3 vitamin; Jones, 1950 (CCl_4 solution, 6.8–7.9 μ; CS_2 solution, 7.9–14.3 μ).

D_4 vitamin.

Epiandrosterone; *see* 3β-hydroxyAndrostan-17-one.

Epiandrosterone, dehydro-; *see* 3β-hydroxy-5-Androsten-17-one.

Epitestosterone; *see* 17α-hydroxy-4-Androsten-3-one.

Equilin; *see* 3β-hydroxy-1,3,5(10),7-Estratetren-17-one.

Equilenin; *see* 3β-hydroxy-1,3,5(10),6,8-Estrapenten-17-one.

5,7,22-Ergostatrien-3β-ol; ergosterol: Breivik, 1954 (CS_2 solution, 8–13 μ); Dobriner, 1953 (No. 53, $CHCl_3$ solution, 5.6–6.3 μ; CCl_4 solution, 6.6–7.7 μ; CS_2 solution, 7.2–12.8 μ); Pirlot, 1948 (CS_2 solution, 8–13 μ); Rosenkrantz, 1952a (crystalline film, 2–16 μ); Turnbull, 1950 (mull, 7.7–15.4 μ).

1,3,5(10)-Estratriene-3,17β-diol; α-estradiol: Carol, 1950 (CS_2 solution, 8.5–14 μ); Furchgott, 1946b (glassy film from pyridine, 2–12.4 μ); Scheer, 1955 (819 $cm.^{-1}$ band).

1,3,5(10)-Estratriene-3,17α-diol; β-estradiol: 3272 CA; Carol, 1950 (CS_2 solution, 8.5–14 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ).

1,3,5(10)-Estratriene-3,16α,17β-triol; estriol: 773 CA; Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Scheer, 1955 (817 $cm.^{-1}$ band).

1,3,5(10)-Estratriene-16,17-dione, 3-hydroxy-; 16-ketoestrone.

1,3,5(10)-Estratrien-17-one, 3-hydroxy-; estrone: 1288 CA; Carol, 1948 (CS_2 solution, 8.5–14 μ); Dobriner, 1953 (No. 5, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Scheer, 1955 (819 $cm.^{-1}$ band).

1,3,5(10),7-Estratetren-17-one, 3β-hydroxy-; equilin: Carol, 1948 (CS_2 solution, 8.5–14 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Scheer, 1955 (807 $cm.^{-1}$ band).

1,3,5(10),6,8-Estrapenten-17-one, 3β-hydroxy-; equilenin: Carol, 1948 (CS_2 solution, 8.5–14 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Scheer, 1955 (816 $cm.^{-1}$ band).

Estriol; *see* 1,3,5(10)-Estratriene-3,16α,17β-triol.

Estrone; *see* 3-hydroxy-1,3,5(10)-Estratrien-17-one.

Estrone, 16-keto-; *see* 3-hydroxy-1,3,5(10)-Estratriene-16,17-dione.

Etiocholane-3α,17α-diol.

Etiocholane-3α,17β-diol: Dobriner, 1953 (No. 71, $CHCl_3$ solution, 5.6–6.2, 8.7–11.5 μ); Rosenkrantz, 1955a (film, 10.0–10.8 μ table).

Etiocholane-3α,16α,17β-triol.

Etiocholane-3,17-dione: Dobriner, 1953 (No. 116, CS_2 solution, 5.6–6.1, 7.2–14.3 μ; CCl_4 solution, 6.7–7.6 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

Etiocholane-3,17-dione, 11β-hydroxy-.

Etiocholane-11,17-dione, 3α-hydroxy-; 11-ketoetiocholanone: Jones, 1949 (CS_2 solution, 8.4–11.2 μ); Rosenkrantz, 1955a (CS_2 solution, 10.0–10.8 μ table).

Etiocholane-3,11,17-trione: Dobriner, 1953 (No. 134, CS_2 solution, 5.6–6.0, 7.2–13.9 μ; CCl_4 solution, 6.7–7.6 μ).

Etiocholan-11-one, 3α,17β-dihydroxy-, diacetate: Dobriner, 1953 (No. 72, CS_2 solution, 5.6–6.0, 7.4–12.9 μ; CCl_4 solution, 6.7–7.5 μ).

Etiocholan-17-one, 3α-hydroxy-; etiocholanolone: Cole, 1954 (solutions, 2.7–14.3 μ); Dobriner, 1948a (CS_2 solution, 2.6–18.2 μ); Dobriner, 1948b (CS_2 solution, 6.8–11.1 μ); Dobriner, 1953 (No. 144, CS_2 solution, 5.6–6.0, 7.2–12.9 μ; CCl_4 solution, 6.7–7.6 μ; No. 298, CCl_4 solution, 2.7–3.6 μ); Furchgott, 1946a (glassy film from pyridine, 2–12.3 μ); Jones, 1948b (CS_2 solution, 6.8–11.2 μ); Jones, 1949 (CS_2 solution, 6.8–11.2 μ); Jones, 1952a (quantitative analysis, CS_2 solution, 5.6–5.9 μ); Jones, 1955a (CS_2 solution, 7.2–14 μ table); Rosenkrantz, 1955a (CS_2 solution, 10.0–10.8 μ table); Rosenkrantz, 1955b (CS_2 solution, 7.5–11.5 μ).

Etiocholan-17-one, 3β-hydroxy-: Cole, 1954 (solutions, 2.7–14.3 μ); Dobriner,

1953 (No. 146, CS_2 solution, 5.6–6.0, 7.2–13.2 μ; CCl_4 solution, 6.7–7.6 μ; No. 297, CCl_4 solution, 2.7 3.6 μ); Jones, 1948b (CS_2 solution, 6.8–11.2 μ); Jones, 1949 (CS_2 solution, 6.8–11.2 μ).

Etiocholan-17-one, 3α,11β-dihydroxy-: 2451 EA-Dobriner, 1953 (No. 168, $CHCl_3$ solution, 5.6–6.0, 8.7–11.8 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

9(11)-Etiocholen-17-one, 3α-hydroxy-: Dobriner, 1953 (No. 148, CS_2 solution, 5.6–6.1, 7.2–12.5 μ; CCl_4 solution, 6.7–7.6 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

Glycocholic acid; cholylglycine.

Lithocholic acid; 3α-hydroxycholanic acid:

methyl ester: 2516 EA-Dobriner, 1953 (No. 233, CS_2 solution, 5.6 6.0, 7.2–14.2 μ; CCl_4 solution, 6.7–7.7 μ); Wooton, 1953 (CS_2 solution, 8.2–11.1 μ).

Pregnane derivatives: *see* Jones, 1952c for 6 μ bands.

Pregnane-3α,20α-diol; pregnanediol: Dobriner, 1953 (No. 77, $CHCl_3$ solution, 5.6–6.1, 8.8–11.8 μ); Furchgott, 1946b (crystalline film from melt, 2–12.4 μ); Rosenkrantz, 1955a (film, 10.0–10.8 μ table).

Pregnane-3α,20β-diol: Rosenkrantz, 1955a (film, 10.0 10.8 μ table).

Pregnane-3β,20α-diol.

Pregnanediol, sodium glucosiduronate of: Furchgott, 1946b (crystalline film from alcohol, 2–11.9 μ).

Pregnane-3α,11β,20α-triol.

Pregnane-3α,17α,20α-triol.

Pregnane-3,20-dione: Dobriner, 1953 (No. 118, CS_2 solution, 5.6 6.0, 7.2 14.3 μ; CCl_4 solution, 6.7 7.6 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

Pregnane-3,20-dione,17α-hydroxy-: 2484 EA-Dobriner, 1953 (No. 201, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ).

Pregnane-3,20-dione,21-hydroxy-, acetate: 2485 EA-Dobriner, 1953 (No. 202, CS_2 solution, 5.6–6.1, 7.2–14.2 μ; CCl_4 solution, 6.7–7.6 μ).

Pregnane-3,20-dione, 11β,21-dihydroxy-.

Pregnane-3,20-dione, 17α,21-dihydroxy-.

Pregnane-3,20-dione, 11β,17α,21-trihydroxy-, 21-acetate: 2503 EA-Dobriner, 1953 (No. 220, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ).

Pregnane-11,20-dione, 3α,21-dihydroxy-.

Pregnane-11,20-dione, 3α,17α,21-trihydroxy-; tetrahydrocortisone; urocortisone: 3α,21-diacetate: 2505 EA-Dobriner, 1953 (No. 222, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ).

Pregnane-3,11,20-trione, 21-hydroxy-.

Pregnane-3,11,20-trione, 17α,21-dihydroxy-.

Pregnan-3α-ol.

Pregnan-11-one, 3α,20α-dihydroxy-: 2456 EA-Dobriner, 1953 (No. 173, $CHCl_3$ solution, 5.6–6.0, 8.7–11.8 μ).

Pregnan-20-one, 3α-hydroxy-; pregnanolone: 763 CA; 2439 EA-Dobriner, 1953 (No. 156, CCl_4 solution, 6.7 7.6 μ; CS_2 solution, 5.6 6.1, 7.2 11.5 μ); Dobriner, 1949 (CS_2 solution, 2.6 5.7, 7.1 11.1 μ); Jones,

1949 (CS_2 solution, 2.8–12.5 μ); Jones, 1955a (CS_2 solution, 7.2–15.0 μ).

Pregnan-20-one, 3α,17α-dihydroxy-; 2462 EA-Dobriner, 1953 (No. 179, $CHCl_3$ solution, 5.6–6.1, 8.7–11.8 μ); Jones, 1949 (CS_2 solution, 8.4–11.2 μ).

Pregnan-20-one, 3α,21-dihydroxy-.

Pregnan-20-one, 3α,11β,17α-trihydroxy-.

Pregnan-20-one, 3α,11β,21-trihydroxy-.

Pregnan-20-one, 3α,17α,21-trihydroxy-.

Pregnan-20-one, 3α,11β,17α,21-tetrahydroxy-; urocortisol: 3,21-diacetate: 2474 EA-Dobriner, 1953 (No. 191, $CHCl_3$ solution, 5.6–6.0, 8.7–11.8 μ).

4-Pregnen-18-al, 11β,21-dihydroxy-3,20-diketo-; aldosterone; electrocortin: 2418 EA-Simpson, 1954 ($CHCl_3$ solution, 2.5–12.1 μ); Harman, 1954 (solid and $CHCl_3$ solution, 2.9–11.5 μ table).

4-Pregnene-3,20-dione; progesterone: 2518 CA; Blout, 1951 (mull and single crystal, 2–15 μ); Dobriner, 1953 (No. 119, CS_2 solution, 5.6–6.1, 7.2–14.3 μ; CCl_4 solution, 6.7–7.6 μ; No. 120, $CHCl_3$, 5.7–6.3, 8.7–11.8 μ; No. 302, CCl_4 solution, 2.7–3.6 μ); Furchgott, 1946b (crystalline film from pyridine, 2–12.4 μ); Jones, 1955a (CS_2 solution, 7.2–14 μ table).

4-Pregnene-3,20-dione, 17α-hydroxy-; 17-hydroxyprogesterone: 2487 EA-Dobriner, 1953 (No. 204, $CHCl_3$ solution, 5.6–6.3, 8.7–11.8 μ).

4-Pregnene-3,20-dione, 21-hydroxy-; deoxycorticosterone: 2142, 2143, 2144 EA-Rosenkrantz, 1953b (melted film, mull, film deposited from $CHCl_3$ and CS_2 solution, 7.5–12 μ); Furchgott, 1947b (glassy film from pyridine, 2–12.4 μ); Hayden, 1955 (KBr disk, 2–15.5 μ).

4-Pregnene-3,20-dione, 11β,21-dihydroxy-; corticosterone: 2141, 2140 EA-Rosenkrantz, 1953b (melted film, mull, film deposited from $CHCl_3$ solution, 7.5–13.5 μ); 2496 EA-Dobriner, 1953 (No. 213, $CHCl_3$ solution, 5.6–6.3, 8.7–11.8 μ); Furchgott, 1947b (crystalline film from pyridine, 2–12.4 μ); Hayden, 1955 (KBr disk, 2–15.5 μ).

4-Pregnene-3,20-dione, 17α,21-dihydroxy-; 11-deoxy-17-hydroxycorticosterone: Hayden, 1955 (KBr disk, 2–15.5 μ).

4-Pregnene-3,20-dione, 11β,17α,21-trihydroxy-; hydrocortisone; cortisol: Antonucci, 1953 (mull, 2–15 μ); Collingsworth, 1953 (mull, 2–15 μ); Hayden, 1955 (KBr disk, 2–15.5 μ); Jones, 1950 (mull, 2.8–3.3, 5.7–14.3 μ).

4-Pregnene-3,11,20-trione, 21-hydroxy-; 11-dehydrocorticosterone: 2508 EA-Dobriner, 1953 (No. 225, $CHCl_3$ solution, 5.6–6.2, 8.7–11.8 μ); Furchgott, 1947b (crystalline film from pyridine, 2–12.4 μ); Hayden, 1955 (KBr disk, 2–15.5 μ).

4-Pregnene-3,11,20-trione, 17α,21-dihydroxy-; cortisone; 17-hydroxy-11-dehydrocorticosterone: Furchgott, 1947b (crystalline film from pyridine, 2–12.4 μ); Hayden, 1955 (KBr disk, 2–15.5 μ); Jones, 1950 (mull, 2.8–3.3, 5.7–14.3 μ).

5-Pregnene-3β,20α-diol.

Progesterone; *see* 4-Pregnene-3, 20-dione.

Progesterone, 17-hydroxy-; *see* 17α-hydroxy-4-Pregnene-3,20-dione.

Sapogenins; *see* Jones, 1953, Dobriner, 1953, and Eddy, 1955 (20-isosapogenin acetates).
Saponins; *see* Rothman, 1952 (mulls and CS_2 solutions, 5.6–6.1, 10–11.7 μ).
Stigmasterol: Rosenkrantz, 1952a (crystalline film, 2–16 μ); Turnbull, 1950 (mull, 7.7–15.4 μ).
Taurocholic acid; cholaic acid; cholyltaurine.
Testosterone; *see* 17β-hydroxy-4-Androsten-3-one.
Urocortisol; *see* 3α,11β,17α,21-tetrahydroxy-Pregnan-20-one.
Urocortisone; *see* 3α,17α,21-trihydroxy-Pregnane-11,20-dione.

Vitamins and Derivatives with up to 2 Atoms Substituted (Except Steroids)

A_1 vitamin: Farrar, 1952 (2.7–14.3 μ).
A vitamins, neo-; 2-*cis*-vitamin A: LB I 464-Robeson, 1947 (6–10.5 μ); Robeson, 1955 (2–15 μ).
A vitamin; "all *trans*" vitamin A: LB I 464-Robeson, 1947 (6–10.5 μ); Robeson, 1955 (2–15 μ).
methyl ether: Oroshnik, 1954 (2–16 μ).
A_2 vitamin.
A vitamin derivatives; retinene and retinene 2: Farrar, 1952 (2.7–14.3 μ).
L-Ascorbic acid; C vitamin: 5424 CA; 1208 EA-Weigl, 1952 (fluorocarbon mull, 2.8–3.8 μ; Nujol mull, 8.8–13.9 μ); LB I 463-Heintz, 1939 (dry film, 1–14 μ; water solution, 1–8 μ); Sternglanz, 1956 (KBr disk and 30 per cent water solution, 2–15 μ); Trotter, 1948 (mull, 2.8–14 μ); Williams, 1937 (water solution, 2–8 μ).
B_{12} vitamin: LB I 464-Barer, 1949 (crystal, 2.8–3.6 μ, 6.3–12.5 μ); Jackson, 1951 (mull, 2.9–14.3 μ).
Benzoic acid, *para*-amino-: 1542 CA.
Biotin.
C vitamin; *see* Ascorbic acid.
Carnitine; B_T vitamin.
Choline.
D vitamins; *see* section on *Steroids*.
E vitamins; *see* under Tocopherols below.
F vitamins; the essential unsaturated fatty acids—only one is required; *see* Arachidonic, Linoleic, and Linolenic acids in section on *Lipides*.
Folic acid; *see N*-pteroyl-L-glutamic acid.
Hesperidin.
Inositols: Barker, 1954c (10.5–13.3 μ table); Shay, 1954 (mull, 6.7–14.3 μ).
i: 1060 CA; LB II 647-Kuhn, 1950 (film from evaporated water solution, 7.8–15 μ).
d: 2778 CA.
K vitamins; *see* under Naphthoquinones below.
1,4-Naphthoquinone, 2-methyl-; menadione, synthetic with vitamin K activity: Rosenkrantz, 1948 (melt, 2–11.8 μ).
1,4-Naphthoquinone, 2-methyl-3-phytyl-; K_1 vitamin: 1912 EA-Isler, 1954 (2–15 μ).
1,4-Naphthoquinone, 2-methyl-3-difarnesyl-: K_2 vitamin.

Niacin.
Nicotinamide: 860 CA.
Nicotinamide, N^1-methyl-.
Nicotinic acid; pyridine-3-carboxylic acid: 434 CA; Flett, 1951 (3.8 11 μ table).
methyl ester: Trenner, 1951 (CS_2 solution, 2–16 μ).
Nicotinuric acid.
P vitamins: *see* Hesperidin, Rutin.
Pantothenic acid,
calcium salt: 524 CA.
2′,4′-diphosphate salt: 1505 EA-Baddiley, 1952 (2–15 μ).
N-Pteroyl-L-glutamic acid: Waller, 1948 (2.7–14.2 μ).
Pyridoxal.
Pyridoxal phosphate.
Pyridoxamine.
Pyridoxamine phosphate.
Pyridoxine: 5992 CA; 8085 CA.
Riboflavin: Barnes, 1944 (p. 99, mull, 5–12.6 μ).
Rutin.
Thiamine, hydrochloride: 324 CA.
Thiamine pyrophosphate.
Tocopherol: 1108 CA (α): 177 EA-Issidorides, 1951 (α,2 16 μ); LB I 464-Rosenkrantz, 1948 (α,γ,δ, liquid, 2–12.1 μ); Boyer, 1951 (*dl*- α, liquid, 2-10 μ); Rosenkrantz, 1950 ($\alpha,\beta,\gamma,\delta,\epsilon$, liquid, 2 16 μ); Stern, 1947 ($\alpha,\beta,\gamma,\delta$, 6–10 μ).

Bibliography

The references marked with an asterisk are utilized in the Introduction only.

Abbott, N. B. & E. J. Ambrose. 1953. Proc. Roy. Soc. London. **A219:** 17–32.
*Abderhalden, E., Ed. 1911-1915. Biochemisches Handlexikon. Springer Verlag. Berlin, Germany.
Adams, R. & V. V. Jones. 1949. J. Am. Chem. Soc. **71:** 3826–3833.
Adams, R. M. & J. J. Katz. 1956. J. Opt. Soc. Am. **46:** 895–898.
Ahamd, K., F. M. Bumpus & F. M. Strong. 1948. J. Am. Chem. Soc. **70:** 3391–3394.
Ahlers, N. H. E., R. A. Brett & N. G. McTaggart. 1953. J. Appl. Chem. London. **3:** 433–443.
Ambrose, E. J. 1950. J. Chem. Soc. : 3246–3249.
Ambrose, E. J., A. Elliott & R. B. Temple. 1951. Proc. Roy. Soc. London **A206:** 192–206.
Anand, N., V. M. Clark, R. H. Hall & A. R. Todd. 1952. J. Chem. Soc. : 3665 3669.
Anderson, D. H. 1953. Anal. Chem. **25:** 1906–1909.
Antonucci, R., S. Bernstein, M. Heller, R. Lenhard, R. Littell & H. Williams. 1953. J. Org. Chem. **18:** 70–82.
Ard, J. S. & T. D. Fontaine. 1951. Anal. Chem. **23:** 133–137.
Astbury, W. T., C. E. Dalgliesh, S. E. Darmon & G. B. B. M. Sutherland. 1948. Nature. **2:** 596–600.
Auméras, M., R. Minangoy, L. Bonnet & B. Laugrost. 1953. Bull. soc. chim. France : 924–927.
Baddiley, J. & E. M. Thain. 1952. J. Chem. Soc. : 3783–3789.
Baer, E., J. Maurukas & M. Russell. 1952. J. Am. Chem. Soc. **74:** 152 157.
Baer, E. 1953. J. Am. Chem. Soc. **75:** 621–623.
Baer, E., D. Buchnea & A. G. Newcombe. 1956. J. Am. Chem. Soc. **78:** 232 237.
Bailey, S. D., P. A. Geary & A. E. de Wald. 1951. J. Am. Pharm. Assoc. Sci. Ed. **40:** 280–286.

Bain, J. P., A. H. Best & R. L. Webb. 1952. J. Am. Chem. Soc. **74:** 4292–4296.
Baird, C. C., T. J. Porro & H. L. Rees. 1955. Anal. Chem. **27:** 12–15.
Baird, W. S., H. M. O'Bryan, G. Ogden & D. Lee. 1947. J. Opt. Soc. Am. **37:** 754–761.
* Baker, A. W., N. Wright & A. Opler. 1953. Anal. Chem. **25:** 1457–1460.
* Bamford, C. H., A. Elliott & W. E. Hanby. 1956. Synthetic Polypeptides: Preparation, Structure, and Properties. Academic Press. New York, N. Y.
Bard, C. C., T. J. Porro & H. L. Rees. 1955. Anal. Chem. **27:** 12–15.
Barer, R., A. R. H. Cole & H. W. Thompson. 1949. Nature. **163:** 198–201.
Barker, S. A., E. J. Bourne, M. Slacey & D. H. Whiffen. 1954a. J. Chem. Soc. : 171–176.
Barker, S. A., E. J. Bourne, R. Stephens & D. H. Whiffen. 1954b. J. Chem. Soc. : 3468–3473.
Barker, S. A., E. J. Bourne, R. Stephens & D. H. Whiffen. 1954c. J. Chem. Soc. : 4211–4215.
Barnes, R. B., U. Liddel & V. Z. Williams. 1943. Ind. Eng. Chem. Anal. Ed. **15:** 659–709.
Barnes, R. B., R. C. Gore, U. Liddel & V. Z. Williams. 1944. Infrared Spectroscopy: Industrial Applications and Bibliography. Reinhold. New York, N. Y.
Barr, E. S. & E. K. Plyler. 1936. J. Chem. Phys. **4:** 90–92.
Barr, E. S. & C. H. Chrisman, Jr. 1940. J. Chem. Phys. **8:** 51–55.
Barr, J. T. & C. A. Horton. 1952. J. Am. Chem. Soc. **74:** 4430–4435.
Barrow, G. M. 1952. J. Chem. Phys. **20:** 1739–1744.
Barrow, G. M. 1953. J. Chem. Phys. **21:** 2008–2011.
Beaman, A. G. 1954. J. Am. Chem. Soc. **76:** 5633–5636.
Becker, R. R. & M. A. Stahmann. 1954. J. Am. Chem. Soc. **76:** 3707–3709.
* Beilstein, F. G. 1918–1956. Handbuch der Organischen Chemie. Fourth edition, with two supplements, covering the literature through 1929. Volume 28: Subject index (with chemical names), 1955. Volume 29: Atomic formula index. 1956. The third supplement, covering the literature through 1949, is in preparation. Springer Verlag. Berlin, Germany.
Bellamy, L. J. 1954. The Infrared Spectra of Complex Molecules. Methuen & Co. London, England.
Benedict, J. H. & B. F. Daubert. 1950. J. Am. Chem. Soc. **72:** 4356–4359.
Bentley, R. R., K. G. Cunningham & F. S. Spring. 1951. J. Chem. Soc. : 2301–2305.
Berger, A. & E. Katchalski. 1951. J. Am. Chem. Soc. **73:** 4084–4088.
Bergmann, E. D., E. Gil-Av & S. Pinchas. 1953. J. Am. Chem. Soc. **75:** 68–71.
Bergstrom, C. G. & S. Siegel. 1952. J. Am. Chem. Soc. **74:** 145–151.
Bickford, W. G., E. F. DuPré, C. H. Mack & R. T. O'Connor. 1953. J. Am. Oil Chemists' Soc. **30:** 376–381.
Bladon, P., J. M. Fabian, H. B. Henbest, H. P. Koch & G. W. Wood. 1951. J. Chem. Soc. : 2402–2411.
Blout, E. R. & M. Fields. 1948. Science. **107:** 252–254.
Blout, E. R. & M. Fields. 1949. J. Biol. Chem. **178:** 335–343.
Blout, E. R., G. R. Bird & D. S. Grey. 1950a. J. Opt. Soc. Am. **40:** 304–313.
Blout, E. R. & M. Fields. 1950b. J. Am. Chem. Soc. **72:** 479–484.
Blout, E. R. & G. R. Bird. 1951. J. Opt. Soc. Am. **41:** 547–551.
Blout, E. R. & S. G. Linsley. 1952a. J. Am. Chem. Soc. **74:** 1946–1951.
Blout, E. R., M. Parrish, Jr., G. R. Bird & M. J. Abbate. 1952b. J. Opt. Soc. Am. **42:** 966–968.
Blout, E. R. & H. Lenormant. 1953. J. Opt. Soc. Am. **43:** 1093–1095.
Blout, E. R. & M. J. Abbate. 1955. J. Opt. Soc. Am. **45:** 1028–1030.
Blout, E. R. & M. Idelson. 1956. J. Am. Chem. Soc. **78:** 497–498.
Bonner, L. G. & R. Hofstadter. 1938. J. Chem. Phys. **6:** 531–534.
Boyer, P. D. 1951. J. Am. Chem. Soc. **73:** 733–740.
Brady, L. J. 1950. Spectrometers: Infrared Region. *In* Analytical Absorption Spectroscopy. M. J. Mellon, Ed. Chap. 8. Wiley & Sons. New York, N. Y.
Breivik, O. N., J. L. Owades & R. F. Ligert. 1954. J. Org. Chem. **19:** 1734–1740.
* Brown, C. R., M. W. Ayton, T. C. Goodwin & T. J. Derby. 1954. Infrared: a Bibliography. Office of Technical Services. Washington, D. C.
Brown, D. M. & A. R. Todd. 1952. J. Chem. Soc. : 44–51.
Brown, J. B., H. B. Henbest & E. R. H. Jones. 1952. J. Chem. Soc. : 3172–3176.
* Brügel, W. 1954. Einführung in die Ultrarotspektroskopie. Steinkopf Verlag. Darmstadt, Germany.

Burch, D. E., J. N. Howard & D. Williams. 1956. J. Opt. Soc. Am. **46:** 452–455.
Buswell, A. M. & R. C. Gore. 1942. J. Phys. Chem. **46:** 575–581.
Cannon, C. G. & G. B. B. M. Sutherland. 1951. Spectrochim. Acta. **4:** 373–395.
Cardwell, H. M. E., J. D. Dunitz & L. E. Argel. 1953. J. Chem. Soc. : 3740–3742.
Carol, J., J. C. Molitor & E. O. Haenni. 1948. J. Am. Pharm. Assoc. Sci. Ed. **37:** 173–179.
Carol, J. 1950. J. Am. Pharm. Assoc. Sci. Ed. **39:** 425–432.
Cason, J., N. K. Freeman & G. Sumrell. 1951. J. Biol. Chem. **192:** 415–424.
Chamberlin, E. M. & J. M. Chemerda. 1955. J. Am. Chem. Soc. **77:** 1221–1223.
Childers, E. & G. W. Struthers. 1955. Anal. Chem. **27:** 737–741.
Chouteau, J. 1953. Bull. soc. chim. France : 1148–1151.
Clark, C. C. 1950a. Infrared Absorption and X Ray Diffraction Identification of Nucleic Acid Type Derivatives. Columbia Univ. Ph.D. thesis. (Publication 1838, University Microfilms, Ann Arbor, Mich.)
* Clark, C. C. 1950b. Science. **111:** 632–633.
Clark, C. C. 1952. Appl. Spectroscopy. **6**(3): 14–17.
Clark, C. C. 1955. Infrared spectrophotometry. *In* Physical Techniques in Biological Research. J. Oster & A. W. Pollister, Ed. **1.** Chap. 5. Academic Press. New York, N. Y.
Coates, V. J., A. Offner & E. H. Siegler, Jr. 1953. J. Opt. Soc. Am. **43:** 984–989.
Coblentz, W. W. 1905- 1906 1908. Investigation of Infrared Spectra. Publications Nos. 35, 65 & 97. Carnegie Institute. Washington, D. C.
Coblentz, W. W. 1953. Appl. Spectroscopy. **7:** 109–111.
Cole, A. R. H., R. N. Jones & K. Dobriner. 1952a. J. Am. Chem. Soc. **74:** 5571–5575.
Cole, A. R. H. & R. N. Jones. 1952b. J. Opt. Soc. Am. **42:** 348–352.
Cole, A. R. H. 1954. Revs. Pure and Appl. Chem. Australia. **4:** 111–132.
Collingsworth, D. R., J. N. Karnemaat, F. R. Hanson, M. P. Brunner, K. M. Mann & W. J. Haines. 1953. J. Biol. Chem. **203:** 807–813.
Conroy, H. 1952. J. Am. Chem. Soc. **74:** 491–498.
Corish, P. J. & W. H. T. Davison. 1955. J. Chem. Soc. : 2431–2436.
Craven, C. W., K. R. Reissmann & H. I. Chinn. 1952. Anal. Chem. **24:** 1214–1215.
Crombie, L. & S. H. Harper. 1950. J. Chem. Soc. : 873–877.
Crombie, L. 1952. J. Chem. Soc. : 2997–3008.
Crombie, L. & J. L. Tayler. 1954. J. Chem. Soc. : 2816–2819.
Crombie, L. 1955. J. Chem. Soc. : 995–1025.
Curcio, J. A. & C. C. Petty. 1951. J. Opt. Soc. Am. **41:** 302–304.
Cymerman, J. & J. B. Willis. 1951. J. Chem. Soc. : 1332–1337.
Darmon, S. E., G. B. B. M. Sutherland & G. R. Tristram. 1948. Biochem. J. **42:** 508–516.
Dauben, W. G., H. L. Bradlow, N. K. Freeman, D. Kritchevsky & M. Kirk. 1952a. J. Am. Chem. Soc. **74:** 4321–4323.
Dauben, W. G. & H. L. Bradlow. 1952b. J. Am. Chem. Soc. **74:** 5204–5206.
Davidson, W. H. J. & G. R. Bates. 1953. J. Chem. Soc. : 2607–2611.
Davies, M. M. 1940. J. Chem. Phys. **8:** 577–587.
Davies, M. & W. J. O. Thomas. 1951. J. Chem. Soc. : 2858–2861.
Davies, M. & J. C. Evans. 1953. J. Chem. Soc. : 480–482.
Davies, M. 1954. J. Chem. Soc. : 121.
Davis, S. B., E. A. Conroy & N. E. Shakespeare. 1950. J. Am. Chem. Soc. **72:** 124–128.
Davoll, J. 1951. J. Am. Chem. Soc. **73:** 3174–3176.
Dekker, C. A. & D. T. Elmore. 1951. J. Chem. Soc. : 2864–2868.
Dekker, C. A. & H. G. Khorana. 1954. J. Am. Chem. Soc. **76:** 3522–3527.
Dobriner, K., S. Lieberman, C. P. Rhoads, R. N. Jones, V. Z. Williams & R. B. Barnes. 1948a. J. Biol. Chem. **172:** 297–311.
Dobriner, K. 1948b. Acta Unio Intern. contra Cancrum. **6:** 315–328.
Dobriner, K., T. H. Kritchevsky, D. K. Fukushima, S. Lieberman, T. F. Gallagher, J. D. Hardy, R. N. Jones & G. Cilento. 1949. Science. **109:** 260–261.
Dobriner, K., E. R. Katzenellenbogen & R. N. Jones. 1953. Infrared Absorption Spectra of Steroids, an Atlas. Interscience. New York, N. Y.
Downie, A. R., M. C. Magoon, T. Purcell & B. Crawford, Jr. 1953. J. Opt. Soc. Am. **43:** 940–951.
* Dreisbach, R. R. 1955. Physical Properties of Chemical Compounds. (511 organic cyclic compounds). American Chemical Society. Washington, D. C.
Duncanson, L. A. 1953. J. Chem. Soc. : 1207–1211.

Duval, C. & J. Lecomte. 1941. Compt. rend. **212:** 389–392.
* Dyson, G. M. 1947. A New Notation and Enumeration System for Organic Compounds. 2nd ed. (1949). Longmans, Green. New York, N. Y.
Ebert, A. A., Jr. & H. B. Gottlieb. 1952. J. Am. Chem. Soc. **74:** 2806–2810.
Eckstein, B. H., H. A. Scheraga & E. R. Van Artsdalen. 1954. J. Chem. Phys. **22:** 28–35.
Eddy, C. R. & A. Eisner. 1954. Anal. Chem. **26:** 1428–1431.
Eddy, C. R., M. A. Barnes & C. S. Fenske. 1955. Anal. Chem. **27:** 1067–1069.
Ellenbogen, E. 1956a. J. Am. Chem. Soc. **78:** 363–366.
Ellenbogen, E. 1956b. J. Am. Chem. Soc. **78:** 366–368.
Ellenbogen, E. 1956c. J. Am. Chem. Soc. **78:** 369–372.
Elliott, A. 1954. Proc. Roy. Soc. London **A226:** 408–421.
* Elsevier's Encyclopedia of Organic Chemistry. 1940–1948. E. Josephy and F. Radt, Eds. 20 vols. **14** (suppl. 1956). Elsevier Publishing Co. New York, N. Y. & Amsterdam, Holland.
Falk, J. E. & J. B. Willis. 1951. J. Sci. Research. **4A:** 579–594.
Farrar, K. R., J. C. Hamlet, H. B. Henbest & E. R. H. Jones. 1952. J. Chem. Soc. : 2657–2668.
Feuge, R. O., M. B. Pepper, Jr., R. T. O'Connor & E. T. Field. 1951. J. Am. Oil Chemists' Soc. **28:** 420–426.
Fichter, R. 1940. Helv. Phys. Acta. **13:** 309–338.
Fletcher, H. G. & H. W. Diehl. 1952. J. Am. Chem. Soc. **74:** 5774–5776.
Flett, M. St. C. 1951. J. Chem. Soc. : 962–967.
Fontaine, T. D., J. S. Ard & R. M. Ma. 1951. J. Am. Chem. Soc. **73:** 878–879.
Fowler, R. G. 1949. Rev. Sci. Instr. **20:** 175–178.
Fowler, R. G. & R. M. Smith. 1953. J. Opt. Soc. Am. **43:** 1054–1057.
Fraser, R. D. B. 1950. Discussions Faraday Soc. **9:** 378–383.
Freeman, N. K. 1953a. J. Am. Chem. Soc. **75:** 1859–1863.
Freeman, N. K., F. T. Lindgren, Y. C. Ng & A. V. Nichols. 1953b. J. Biol. Chem. **203:** 293–304.
Freeman, N. K. 1956. Advances in Biol. and Med. Phys. **4:** 167–221.
French, R. O., M. E. Wadsworth, M. A. Cook & I. B. Cutler. 1954. J. Phys. Chem. **58:** 805–811.
Friedel, R. A. 1951. J. Am. Chem. Soc. **73:** 2881–2884.
Furchgott, R. F., H. Rosenkrantz & E. Shorr. 1946a. J. Biol. Chem. **163:** 375–386.
Furchgott, R. F., H. Rosenkrantz & E. Shorr. 1946b. J. Biol. Chem. **164:** 621–630.
Furchgott, R. F., H. Rosenkrantz & E. Shorr. 1947a. J. Biol. Chem. **167:** 627–635.
Furchgott, R. F., H. Rosenkrantz & E. Shorr. 1947b. J. Biol. Chem. **171:** 523–529.
Fürst, A., H. H. Kuhn, R. Scotoni, Jr. & Hs. H. Günthard. 1952. Helv. Chim. Acta. **35:** 951–957.
Fusari, S. A., R. P. Frohardt, A. Ryder, T. H. Haskell, D. W. Johannessen, C. C. Elder & Q. R. Bartz. 1954. J. Am. Chem. Soc. **76:** 2878–2881.
Gäumann, T. & Hs. H. Günthard. 1952. Helv. Chim. Acta. **35:** 53–60.
Geissman, T. A. & A. Armen. 1952. J. Am. Chem. Soc. **74:** 3916–3919.
Gore, R. C., R. B. Barnes & E. Petersen. 1949. Anal. Chem. **21:** 382–386.
Grob, C. A. & E. F. Jenny. 1952. Helv. Chim. Acta. **35:** 2106–2111.
Gupta, S. D. & J. S. Aggarwal. 1955. J. Am. Oil Chemists' Soc. **32:** 501–503.
Guy, J. 1949. Bull. soc. chim. France. : 731–742.
Hacskaylo, M. 1954. Anal. Chem. **26:** 1410–1412.
Hadzi, D. & N. Sheppard. 1953. Proc. Roy. Soc. London. **A216:** 247–266.
Haines, W. E., R. V. Helm, C. W. Bailey & J. S. Ball. 1954. J. Phys. Chem. **58:** 270–278.
Hanahan, D. J. & M. E. Jayco. 1952. J. Am. Chem. Soc. **74:** 5070–5073.
Hanahan, D. J., M. Rodbell & L. D. Turner. 1954a. J. Biol. Chem. **206:** 431–441.
Hanahan, D. J. & R. Vercamer. 1954b. J. Am. Chem. Soc. **76:** 1804–1806.
Harman, R. E., E. A. Ham, J. E. DeYoung, N. G. Brink & L. H. Sarett. 1954. J. Am. Chem. Soc. **76:** 5035–5036.
Harple, W. H., S. E. Wiberley & W. H. Bauer. 1952. Anal. Chem. **24:** 635–638.
Harris, R. J. C., S. F. D. Orr, E. M. F. Roe & J. F. Thomas. 1953. J. Chem. Soc. : 489–494.
Hausdorff, H. 1954. Appl. Spectroscopy **8:** 131–136.
Hayden, A. L. 1955. Anal. Chem. **27:** 1486–1489.
* Heilbron, I. M. & H. M. Bunbury. 1953. Dictionary of Organic Compounds. 4 vols. Oxford Univ. Press. New York, N. Y.

Heintz, E. 1939. Compt. rend. **208:** 1893–1896.
Hergert, H. L. & E. F. Kurth. 1953. J. Org. Chem. **18:** 521–529.
Herman, R. C. & R. Hofstadter. 1938. J. Chem. Phys. **6:** 534–540.
Herman, R. C. & R. Hofstadter. 1939. J. Chem. Phys. **7:** 460–464.
Herman, R. C. 1940. J. Chem. Phys. **8:** 252–258.
* Hershenson, H. M. 1956. Ultraviolet and Visible Absorption Spectra. (Literature index, 1930–1954.) Academic Press. New York, N. Y.
Hilbert, G. E., O. R. Wulf, S. B. Hendricks & U. Liddel. 1936. J. Am. Chem. Soc. **58:** 548–555.
Hinman, J. W., E. L. Caron & H. N. Christensen. 1950. J. Am. Chem. Soc. **72:** 1620–1626.
Hofmann, K., R. A. Lucas & S. M. Sax. 1952. J. Biol. Chem. **195:** 473–485.
Hofmann, K. & S. M. Sax. 1953. J. Biol. Chem. **205:** 55–63.
Hofmann, K., O. Jucker, W. R. Miller, A. C. Young, Jr. & F. Tausig. 1954. J. Am. Chem. Soc. **76:** 1799–1804.
* Hoppe-Seyler Thierfelder. 1955. Handbuch der physiologisch- und pathologisch-chemischen Analyse. 10th ed. Springer Verlag. Berlin, Germany.
Hornberger, C. S., Jr., R. F. Heitmiller, I. C. Gunsalus, G. H. F. Schnakenberg & L. J. Reed. 1953. J. Am. Chem. Soc. **75:** 1273–1277.
Howard, G. A. & J. R. A. Pollock. 1952. J. Chem. Soc. : 1902–1906.
Howard, J. N., D. E. Burch & D. Williams. 1956a. J. Opt. Soc. Am. **46:** 186–190.
Howard, J. N., D. E. Burch & D. Williams. 1956b. J. Opt. Soc. Am. **46:** 237–241.
Howard, J. N., D. E. Burch & D. Williams. 1956c. J. Opt. Soc. Am. **46:** 242–245.
Howe, R. & F. J. McQuillin. 1955. J. Chem. Soc. : 2423–2428.
Howton, D. R. & R. H. Davis. 1951. J. Org. Chem. **16:** 1405–1413.
* Hunt, J. M., M. P. Wisherd & L. C. Bonham. 1950. Anal. Chem. **22:** 1478–1497.
Hurd, C. D., L. Bauer & I. M. Klotz. 1953. J. Am. Chem. Soc. **75:** 624–626.
Idler, D. R. & C. A. Baumann. 1952. J. Biol. Chem. **195:** 623–628.
Isler, O. & K. Doebel. 1954. Helv. Chim. Acta. **37:** 225–233.
Issidorides, A. 1951. J. Am. Chem. Soc. **73:** 5146–5148.
Jackson, W. G., G. B. Whitfield, W. H. DeVries, H. A. Nelson & J. S. Evans. 1951. J. Am. Chem. Soc. **73:** 337–341.
Johnson, D. R., D. R. Idler, V. W. Meloche & C. A. Baumann. 1953. J. Am. Chem. Soc. **75:** 52–55.
Johnson, J. L., W. G. Jackson & T. E. Eble. 1951. J. Am. Chem. Soc. **73:** 2947–2948.
Jones, L. A., C. Holmes & R. B. Seligman. 1956. Anal. Chem. **28:** 191–198.
Jones, L. H. & E. McLaren. 1954. J. Chem. Phys. **22:** 1796–1800.
Jones, R. N., V. Z. Williams, M. J. Whalen & K. Dobriner. 1948a. J. Am. Chem. Soc. **70:** 2024–2034.
Jones, R. N. 1948b. Recent Progr. in Hormone Research. **2:** 3–29.
Jones, R. N. & K. Dobriner. 1949. Vitamins and Hormones. **7:** 293–363.
Jones, R. N. 1950. Chem. in Can. **2:** 94–98.
Jones, R. N., D. A. Ramsay, D. S. Keir & K. Dobriner. 1952a. J. Am. Chem. Soc. **74:** 80–88.
Jones, R. N., A. F. McKay & R. G. Sinclair. 1952b. J. Am. Chem. Soc. **74:** 2575–2578.
Jones, R. N., P. Humphries, F. Herling & K. Dobriner. 1952c. J. Am. Chem. Soc. **74:** 2820–2828.
Jones, R. N., E. Katzenellenbogen & K. Dobriner. 1953. J. Am. Chem. Soc. **75:** 158–166.
Jones, R. N., F. Herling & E. Katzenellenbogen. 1955a. J. Am. Chem. Soc. **77:** 651–661.
Jones, R. N., B. Nolin & G. Roberts. 1955b. J. Am. Chem. Soc. **77:** 6331–6340.
Jones, R. N. & C. Sandorfy. 1956. The application of infrared and Raman spectrometry to the elucidation of molecular structure. *In* Chemical Applications of Spectroscopy. W. West, Ed. Chap. 4. Interscience. New York, N. Y.
Josien, M. L., N. Fuson & A. S. Cary. 1951. J. Am. Chem. Soc. **73:** 4445–4449.
Keller, W. E. 1948. J. Chem. Phys. **16:** 1003–1004.
Kellner, L. 1941. Proc. Roy. Soc. London. **A117:** 447–456.
Kendall, D. N., R. R. Hampton & H. Hausdorff. 1953a. Appl. Spectroscopy. **7:** 179–196.
* Kendall, D. N. 1953b. Anal. Chem. **25:** 382–389.
* King, G. W. & A. G. Emslie. 1953. J. Opt. Soc. Am. **43:** 664–668.
* King, G. W., E. H. Blanton & J. Frawley. 1954. J. Opt. Soc. Am. **44:** 397–402.

* Kirk, R. E. & D. F. Othmer. 1950. Encyclopedia of Chemical Technology. Interscience. New York, N. Y.
Klotz, I. M. & D. M. Gruen. 1948. J. Phys. & Colloid Chem. **52:** 961–968.
Klotz, I. M., P. Griswold & D. M. Gruen. 1949. J. Am. Chem. Soc. **71:** 1615–1620.
Koegel, R. J., J. P. Greenstein, M. Winitz, S. M. Birnbaum & R. A. McCallum. 1955. J. Am. Chem. Soc. **77:** 5708–5720.
Kornfeld, E. C., E. J. Fornefeld, G. B. Kline, M. J. Mann, D. E. Morrison, R. G. Jones & R. B. Woodward. 1956. J. Am. Chem. Soc. **78:** 3087–3114.
* Kostowski, H. J. & A. M. Bass. 1956. J. Opt. Soc. Am. **46:** 1060–1064.
Krimm, S. 1955. J. Chem. Phys. **23:** 1371–1372.
* Kuentzel, L. E. 1951. Anal. Chem. **23:** 1413–1418.
Kuhn, L. P. 1950. Ind. Eng. Chem. Anal. Ed. **22:** 276–283.
Kuhn, W. & H. Schinz. 1952. Helv. Chim. Acta. **35:** 2008–2015.
Kuhrt, N. H., E. A. Welch, W. P. Blum, E. S. Perry, W. H. Weber & E. S. Nasset. 1952. J. Am. Oil Chemists' Soc. **29:** 261–278.
Lacher, J. R., V. D. Croy, A. Kianpour & J. D. Park. 1954. J. Phys. Chem. **58:** 206–210.
Landolt-Börnstein. 1951. Zahlenwerte und Funktionen aus Physik, Chemie, Astronomie, Geophysik, und Technik. 6th ed. Molekeln I (Kerngerüst). **1:** Part 2. Molekeln II (Elektronenhülle). **1** : Part 3. Springer Verlag. Berlin, Germany.
Larsson, L. 1950. Acta. Chem. Scand. **4:** 27–38.
Laubengayer, A. W. & W. C. Smith. 1954. J. Am. Chem. Soc. **76:** 5985–5989.
Lecomte, J., T. Pobeguin & J. Wyart. 1943. Compt. rend. **216:** 808–810.
Lenormant, H. 1945. Compt. rend. **221:** 545–547.
Lenormant, H. 1946. Compt. rend. **222:** 1432–1434.
Lenormant, H. 1952a. Compt. rend. **234:** 1959.
Lenormant, H. & J. Couteau. 1952b. Compt. rend. **234:** 2057–2059.
Lenormant, H. & E. R. Blout. 1954. Compt. rend. **239:** 1281–1283.
* Li, S. O. & R. E. Eakin. 1955. J. Am. Chem. Soc. **77:** 3519–3521.
Lieberman, S., L. B. Hariton & D. K. Fukushima. 1948. J. Am. Chem. Soc. **70:** 1427–1432.
MacDonald, S. F. 1952. J. Chem. Soc. : 4184–4190.
Mann, J. & H. W. Thompson. 1948. Proc. Roy. Soc. London. **A192:** 489–497.
Manning, J. J. 1956. Appl. Spectroscopy. **7:** 85–98.
Margoshes, M., F. Fillwalk, V. A. Fassel & R. E. Rundle. 1954. J. Chem. Phys. **22:** 381–382.
Marinetti, G. & E. Stotz. 1954. J. Am. Chem. Soc. **76:** 1347–1352.
Mason, S. F. 1955. J. Chem. Soc. : 2336–2346.
Michelson, A. M. & A. R. Todd. 1954. J. Chem. Soc. : 34–40.
* Miller, F. A. & C. H. Wilkins. 1952. Anal. Chem. **24:** 1253–1294.
Mills, I. M., J. R. Scherer, B. Crawford, Jr. & M. Youngquist. 1955. J. Opt. Soc. Am. **45:** 785–787.
Mislow, K. 1952. J. Am. Chem. Soc. **74:** 5155–5157.
Mold, J. D., R. C. Gore, J. M. Lynch & E. J. Schantz. 1955. J. Am. Chem. Soc. **77:** 178–180.
Moore, J. A., J. D. Dice, E. D. Nicolaides, R. D. Westland & E. L. Wittle. 1954. J. Am. Chem. Soc. **76:** 2884–2887.
* Morgan, J. A. & D. E. H. Frear. 1947. J. Chem. Educ. **24:** 58–61.
Morris, J. C. 1943. J. Chem. Phys. **11:** 230–235.
Murray, J. 1954. J. Am. Chem. Soc. **76:** 3665.
Naves, Y.-R. & J. Lecomte. 1953. Bull. soc. chim. France. : 112–124.
Neuilly, M. 1954. Compt. rend. **238:** 65–67.
Nielsen, H. H. & R. A. Oetjen. 1950. Infrared spectroscopy. *In* Physical Methods in Chemical Analysis. W. G. Berl, Ed. **1:** 333–404. Academic Press. New York, N. Y.
O'Connor, R. T., E. T. Field & W. S. Singleton. 1951. J. Am. Oil Chemists' Soc. **28:** 154–160.
O'Connor, R. T., P. Von der Haar, E. F. DuPré, L. E. Brown & C. H. Pominski. 1954a. J. Am. Chem. Soc. **76:** 2368–2373.
O'Connor, R. T. & L. A. Goldblatt. 1954b. Anal. Chem. **26:** 1726–1737.
O'Connor, R. T., E. F. DuPré & R. O. Feuge. 1955. J. Am. Oil Chemists' Soc. **32:** 88–93.
O'Connor, R. T. 1956. J. Am. Oil Chemists' Soc. **33:** 1–15.
Oetjen, R. A., W. H. Haynie, W. M. Ward, R. L. Hansler, H. E. Schauwecker & E. E. Bell. 1952. J. Opt. Soc. Am. **42:** 559–566.
* Opler, A. & T. R. Norton. 1956. Chem. Eng. News. **34:** 2812–2816.

Oroshnik, W. 1954. J. Am. Chem. Soc. **76:** 5499–5505.
Orr, S. F. D., R. J. C. Harris & B. Sylven. 1952. Nature. **169:** 544–545.
Orr, S. F. D. 1954. Biochim. et Biophys. Acta. **14:** 173–181.
Osberg, W. E. & D. F. Hornig. 1952. J. Chem. Phys. **20:** 1345–1347.
Otey, M. C. & J. P. Greenstein. 1954. Arch. Biochem. Biophys. **53:** 501–513.
Parke, T. V. 1951. Anal. Chem. **23:** 953–957.
Pierson, R. H., A. N. Fletcher & E. S. Glantz. 1956. Anal. Chem. **28:** 1218–1239.
Pirlot, G. 1948. Anal. Chim. Acta. **2:** 744–749.
Pitzer, K. S. & W. Weltner, Jr. 1949. J. Am. Chem. **71:** 2842–2844.
Plyler, E. K. & C. J. Humphreys. 1947. J. Research Natl. Bur. Standards. **39:** 59–65.
Plyler, E. K. & N. Acquista. 1953. J. Opt. Soc. Am. **43:** 212.
Plyler, E. K. & N. Acquista. 1954. J. Opt. Soc. Am. **44:** 505–506.
* Powell, H. 1952. Appl. Spectroscopy. **6**(2): 3–7.
Price, D. 1941. J. Chem. Phys. **9:** 725–726.
Pristera, F. 1952. Appl. Spectroscopy. **6**(3): 29–44.
Pristera, F. 1953. Anal. Chem. **25:** 844–856.
Pristera, F. & A. Castelli. 1955. Anal. Chem. **27:** 457–459.
* Ramachandran, L. K., A. Epp & W. B. McConnell. 1955. Anal. Chem. **27:** 1734–1737.
Randall, H. M., R. G. Fowler, N. Fuson & J. R. Dangl. 1949. Infrared Determination of Organic Structures. Van Nostrand. New York, N. Y.
Rao, P. C. & B. F. Daubert. 1948. J. Am. Chem. Soc. **70:** 1102–1104.
Rasmussen, R. S. & R. R. Brattain. 1949. J. Am. Chem. Soc. **71:** 1073–1079.
Rigaux, C. 1954. Compt. rend. **238:** 63–65.
Roberts, J. D., D. R. Smith & C. C. Lee. 1951. J. Am. Chem. Soc. **73:** 618–625.
Robeson, C. D. & J. G. Baxter. 1947. J. Am. Chem. Soc. **69:** 136–141.
Robeson, C. D., J. D. Cawley, L. Weisler, M. H. Stern, C. C. Eddinger & A. J. Chechak. 1955. J. Am. Chem. Soc. **77:** 4111–4119.
Rogers, L. H. & D. Williams. 1938. J. Am. Chem. Soc. **60:** 2619–2621.
Rosenkrantz, H. 1948. J. Biol. Chem. **173:** 439–447.
Rosenkrantz, H. & A. T. Milhorat. 1950. J. Biol. Chem. **187:** 83–90.
Rosenkrantz, H., A. T. Milhorat & M. Farber. 1952a. J. Biol. Chem. **195:** 503–507.
Rosenkrantz, H., A. T. Milhorat & M. Farber. 1952b. J. Biol. Chem. **195:** 509–514.
Rosenkrantz, H. & L. Zablow. 1953a. J. Am. Chem. Soc. **75:** 903–907.
Rosenkrantz, H. & L. Zablow. 1953b. Anal. Chem. **25:** 1025–1028.
Rosenkrantz, H. & P. Skogstrom. 1955a. J. Am. Chem. Soc. **77:** 2237–2241.
Rosenkrantz, H. 1955b. Analysis of steroids by infrared spectrometry. *In* Methods of Biochemical Analysis. D. Glick, Ed. **2.** Chap. 1. Interscience. New York, N. Y.
* Rossini, F. D. 1951. Appl. Spectroscopy. **6**(1): 3–13.
Rothman, E. S., M. E. Wall & C. R. Eddy. 1952. J. Am. Chem. Soc. **74:** 4013–4016.
Scheer, I., W. R. Nes & P. B. Smeltzer. 1955. J. Am. Chem. Soc. **77:** 3300–3305.
Schneider, W. G. & H. J. Bernstein. 1956. Trans. Faraday Soc. **52:** 13–18.
Schönmann, E. 1943. Helv. Phys. Acta. **16:** 343–364.
Schwarz, H. P. 1952. Appl. Spectroscopy. **6**(4): 15–18.
Scott, J. F., R. I. Sinsheimer & J. R. Loofbourow. 1952. J. Am. Chem. Soc. **74:** 275–277.
Shay, J. F., S. Skilling & R. W. Stafford. 1954. Anal. Chem. **26:** 652–656.
Sheppard, N. & G. B. B. M. Sutherland. 1945. Trans. Faraday Soc. **41:** 261–279.
Sheppard, N. 1949. J. Chem. Phys. **17:** 79–83.
Sheppard, N. 1950. Trans. Faraday Soc. **46:** 429–439.
Short, L. N. & H. W. Thompson. 1951. J. Chem. Soc. : 1746–1749.
Short, L. N. & H. W. Thompson. 1952. J. Chem. Soc. : 168–187.
Shreve, O. D., M. R. Heether, H. B. Knight & D. Swern. 1950. Anal. Chem. **22:** 1498–1501.
Shreve, O. D. 1952. Anal. Chem. **24:** 1692–1699.
Simpson, S. A., J. F. Tait, A. Wettstein, R. Neher, J. v. Euw, O. Schindler & T. Reichstein. 1954. Helv. Chim. Acta. **37:** 1163–1200.
Sinclair, R. G., A. F. McKay & R. N. Jones. 1952a. J. Am. Chem. Soc. **74:** 2570–2575.
Sinclair, R. G., A. F. McKay, G. S. Myers & R. N. Jones. 1952b. J. Am. Chem. Soc. **74:** 2578–2585.
Smith, F. A. & E. C. Creitz. 1951. J. Research Natl. Bur. Standards. **46:** 145–164.
* Smith, L. C. 1955. Library J. **80:** 23–27.
Smith, W. M., Jr., K. C. Eberley, E. E. Hanson & J. L. Binder. 1956. J. Am. Chem. Soc. **78:** 626–630.
* Spector, W. S., Ed. 1956. Handbook of Biological Data. Division of Biology and

Agriculture, National Academy of Sciences, National Research Council. Saunders. Philadelphia, Pa.
Speeter, M. E., R. V. Heinzelmann & D. I. Weisblat. 1951. J. Am. Chem. Soc. **73:** 5514–5515.
Ställberg-Stenhagen, S., E. Stenhagen, N. Sheppard, G. B. B. M. Sutherland & A. Walsh. 1947. Nature. **160:** 580.
Stern, M. H., C. D. Robeson, L. Weisler & J. G. Baxter. 1947. J. Am. Chem. Soc. **69:** 869–874.
Sternglanz, H. 1956. Appl. Spectroscopy. **10:** 77–82.
Stimson, M. M. & M. J. O'Donnell. 1952. J. Am. Chem. Soc. **74:** 1805–1808.
Sutherland, G. B. B. M. 1952. Advances in Protein Chem. **7:** 291–318.
Swern, D., L. P. Whitnauer, C. R. Eddy & W. E. Parker. 1955. J. Am. Chem. Soc. **77:** 5537–5541.
Tallan, H. H., W. H. Stein & S. Moore. 1954. J. Biol. Chem. **206:** 825–834.
Thomas, L. C. 1955. Nature. **175:** 424–425.
Thompson, H. W. & N. P. Skerrett. 1940. Trans. Faraday Soc. **36:** 812–817.
Thompson, H. W. & G. P. Harris. 1942. Trans. Faraday Soc. **38:** 37–46.
Thompson, H. W. & P. Torkington. 1945. Trans. Faraday Soc. **41:** 246–260.
Thompson, H. W., D. L. Nicholson & L. N. Short. 1950. Discussions Faraday Soc. **9:** 222–235.
* Thompson, H. W. 1955. J. Chem. Soc. **:** 4501–4509.
Tipson, R. S. & L. H. Cretcher. 1951. J. Org. Chem. **16:** 1091–1099.
Tipson, R. S. & M. A. Clapp. 1953. J. Org. Chem. **18:** 952–963.
Toribara, T. Y. & V. DiStefano. 1954. Anal. Chem. **26:** 1519–1521.
Torkington, P. 1945. Trans. Faraday Soc. **41:** 184–186.
Trenner, N. R., R. W. Walker, B. Arison & C. Trumbauer. 1951. Anal. Chem. **23:** 487–490.
Trotter, I. F. & H. W. Thompson. 1946. J. Chem. Soc. **:** 481–488.
Trotter, I. F., H. W. Thompson & F. Wokes. 1948. Biochem. J. **42:** 601–602.
Turnbull, J. H., D. H. Whiffen & W. Wilson. 1950. Chem. & Ind. London. **:** 626.
Ultee, A. J., Jr. & J. Hartel. 1955. Anal. Chem. **27:** 557–560.
Vafiadi, V. G. 1938. Zhur. Obshcheĭ Khim. **8:** 1447–1453.
Verne, J., J. Lecomte & R. Wegmann. 1955. Arch. Biol. Med. **31:** 1–8.
Vestling, C. S. & J. R. Downing. 1939. J. Am. Chem. Soc. **61:** 3511–3513.
Walborsky, H. M., R. H. Davis & D. R. Howton. 1951. J. Am. Chem. Soc. **73:** 2590–2594.
Waldron, R. D. & R. M. Badger. 1950. J. Chem. Phys. **18:** 566.
Wall, F. T. & W. F. Chaussen. 1939. J. Am. Chem. Soc. **61:** 2812–2815.
Waller, C. W., B. L. Hutchings, J. H. Mowat, E. L. R. Stokstad, J. H. Boothe, R. B. Angier, J. Semb, Y. SubbaRow, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels & J. M. Smith, Jr. 1948. J. Am. Chem. Soc. **70:** 19–22.
Wang, S. C. & J. P. Hummel. 1952. J. Am. Chem. Soc. **74:** 2445.
Warnell, J. L. & C. P. Berg. 1954. J. Am. Chem. Soc. **76:** 1708–1709.
Wehrli, M. & R. Fichter. 1941. Helv. Phys. Acta. **14:** 189–194.
Weigl, J. W. 1952. Anal. Chem. **24:** 1483–1486.
Weigl, J. W. & R. Livingston. 1953. J. Am. Chem. Soc. **75:** 2173–2176.
Whiffen, D. H. & H. W. Thompson. 1945. J. Chem. Soc. **:** 268–273.
White, J. U. 1950. Anal. Chem. **22:** 768–772.
Williams, D. & L. H. Rodgers. 1937. J. Am. Chem. Soc. **59:** 1422–1423.
Williams, V. Z. 1947. J. Chem. Phys. **15:** 232–242.
* Winchell, A. N. 1954. The Optical Properties of Organic Compounds. 2nd ed. Academic Press. New York, N. Y.
Witkop, B. & J. B. Patrick. 1953. J. Am. Chem. Soc. **75:** 2572–2576.
Witkop, B. & T. W. Beiler. 1954a. J. Am. Chem. Soc. **76:** 5589–5597.
Witkop, B. 1954b. J. Am. Chem. Soc. **76:** 5597–5599.
Woernley, D. L. 1952. Cancer Research. **12:** 516–523.
* Wood, G. C. 1956. A Detailed Biology Code for Storing, Retrieving, and Correlating Chemical-Biological Data. Chemical Biological Coordination Center, National Academy of Sciences National Research Council. American Documentation. Washington, D. C. In press.
Woodward, R. B., F. Sondheimer, D. Taub, K. Heusler & W. M. McLamore. 1952. J. Am. Chem. Soc. **74:** 4223–4251.
Wooton, I. D. P. 1953. Biochem. J. **53:** 85–88.

Wright, N. 1937. J. Biol. Chem. **120:** 641–646.
Wright, N. 1939. J. Biol. Chem. **127:** 137–141.
Wright, N. & L. W. Herscher. 1947. J. Opt. Soc. Am. **37:** 211–216.
Wright, N. 1955. Appl. Spectroscopy. **9:** 105–118.
Wulf, O. R., U. Liddell & S. B. Hendricks. 1936. J. Am. Chem. Soc. **58:** 2287–2293.
* Wyckoff, R. W. G. 1948, 1951, 1953. Crystal Structures. (Three volumes with supplements.) Interscience. New York, N. Y.
Zacharius, R. M., J. F. Thompson & F. C. Steward. 1954. J. Am. Chem. Soc. **76:** 2908–2912.

Printed in the United States of America

MONOGRAPHIC PUBLICATIONS

OF

THE NEW YORK ACADEMY OF SCIENCES

(LYCEUM OF NATURAL HISTORY, 1817–1876)

(1) The ANNALS (octavo series), established in 1823, contain the scientific contributions and reports of researches, together with the records of meetings of the Academy. The articles that comprise each volume are printed separately, each in its own cover, and are distributed immediately upon publication. The prices of the separate articles depend upon their length and the number of illustrations, and may be ascertained upon application to the Executive Director of the Academy.

Current numbers of the ANNALS are sent free to all Members of the Academy desiring them.

(2) The SPECIAL PUBLICATIONS, established in 1939, are issued at irregular intervals as cloth-bound volumes. The price of each volume will be advertised at time of issue.

(3) The MEMOIRS (quarto series), established in 1895, are issued at irregular intervals. It is intended that each volume shall be devoted to monographs relating to some particular department of science. Volume I, Part 1 is devoted to Astronomical Memoirs, Volume II to Zoological Memoirs. No more parts of the Memoirs have been published to date. The price is one dollar per part.

(4) The SCIENTIFIC SURVEY OF PORTO RICO AND THE VIRGIN ISLANDS (octavo series), established in 1919, gives the detailed reports of the anthropological, botanical, geological, paleontological, zoological, and meteorological surveys of these islands.

Subscriptions and inquiries concerning current and back numbers of any of the publications of the Academy should be addressed to

EXECUTIVE DIRECTOR
The New York Academy of Sciences
2 East Sixty-third Street
New York 21, N. Y.